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Induction of MLR-Bf and protection of fetal loss: a current double blind randomized trial of paternal lymphocyte immunization for women with recurrent spontaneous abortion

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Abstract

The present study was conducted to evaluate the efficacy of paternal lymphocyte (PL) immunotherapy and its relation with the development of mixed lymphocyte reaction blocking antibodies (MLR-Bf) and the success of pregnancy outcome in women with recurrent spontaneous abortion (RSA). A total of 124 women with unknown causes of abortions was registered for immunotherapy under double blind randomized trial by using the list of computer-generated numbers. Each 5×10^6 autologous lymphocyte (AL), third party lymphocyte (TPL) and PL was dissolved separately in 1 ml of sterile normal saline (NS). Each 1 ml of cell suspension and neat NS was injected in women with RSA through intramuscular (250 μl), intradermal (250 μl), subcutaneous (250 μl) and intravenous (250 μl) routes. All women participants with RSA received six identical immunizations at the regular interval of 4 weeks, and were then screened for the development of MLR-Bf after the completion of immunization course, and also at the first, second and third trimesters (12th, 24th and 36th weeks) of pregnancy. However, nonimmunized MLR-Bf positive women with RSA did not receive any kind of therapy (NT) and were used as one of the control group in the present study. We have observed that PL-immunized women with RSA showed a significantly increased level of MLR-Bf (>30) and pregnancy success (84%) as compared to those women with RSA who received either AL (33%), TPL (31%), NS (25%) or those who did not receive any kind of treatment (NT, 44%; $P < 0.001$). Our results indicated the importance of immunotherapy with PL in women with RSA and also showed that MLR-Bf can be considered as one of the important factors for pregnancy improvement.

Keywords: Recurrent spontaneous abortion; Mixed lymphocyte reaction blocking antibodies; Lymphocyte immunotherapy

1. Introduction

First trimester miscarriage is the commonest complication of pregnancy affecting approximately 1 in 300 pregnant women [1,2]. Recurrent spontaneous abortion (RSA) can be defined as the occurrence of three or more clinically detectable pregnancy failure before 20 week of gestation from the last menstrual period or less than 500 g of fetal body weight [3–5]. In the vast majority of the cases, the etiology is unknown and several hypotheses have been proposed on the basis of available data. These have varied from genetic [6,7], anatomical [8–10], endocrine [11],

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placental anomalies [12–14], smoking and alcohol consumption [15], exposure to environmental factors such as lead, mercury, ethylene oxide and ionizing radiations [16], to immunological factors [17–27]. RSA can be classified into primary RSA aborters and secondary RSA aborters. Primary RSA aborters are those who have lost all previous pregnancies and have no live birth. Secondary RSA aborters are those who have at least one successful pregnancy, irrespective of the number of pregnancy losses. Epidemiological studies suggested that the risk of subsequent pregnancy loss is approximately 24% after two clinical pregnancy losses, 30% after three and 40% after four consecutive spontaneous abortions [28,29].

There are evidences which revealed that immunological recognition of pregnancy is important for the maintenance of gestation, and inadequate recognition of fetal antigens might lead to abortion in women with RSA [30,31]. Alloimmunity has been indicated in several studies by showing an association of habitual abortion with an increased sharing of human leukocyte antigens (HLA) with the father that may prohibit the production of antipaternal cytotoxic antibodies (APCA), anti-idiotypic antibodies (Ab2) and mixed lymphocyte reaction blocking antibodies (MLR-Bf) in the mother. Paternal lymphocyte (PL) immunization is an effective treatment for unexplained recurrent spontaneous abortions, as it was attributed to the production of APCA [17,22,32–34], Ab2 [21,35,36] and MLR-Bf [2,17,20,26,27,31,32,37–45] during pregnancy in women with RSA. Some of the recent studies demonstrated that PL immunotherapy significantly reduced the overactivity of NK cells and Th-1 pattern of cytokines in women with RSA [46–48]. However, our earlier studies have shown that the efficacy of lymphocyte immunotherapy was related to immune response to allogenic lymphocytes [17,24]. We further demonstrated that MLR-Bf developed during pregnancy and alloimmunotherapy was IgG 3 in nature [26]. The purpose of this double-randomized trial was to reconfirm the efficacy of immunization with PL as a treatment for unknown causes of women with RSA.

2. Methods

A total of 124 women with RSA of unknown causes was registered for PL immunotherapy under double blind randomized trial. All of these women were negative for abnormal karyotyping, serology for toxoplasma, antiphospholipid antibodies, antinuclear antibodies, antithyroid antibodies, antiendothelial cell antibodies, glucose tolerance test, hysterosalpingogram, thyroid function test, luteal phase plasma progesterone concentrations, pelvic USG and mixed lymphocyte reaction blocking antibodies (MLR-Bf). The details of the protocol approved by the ethical committee of the institute were explained to all eligible couples, and only those who gave written informed consent were included in the study.

2.1. Double blind randomization of women with RSA

All women participants with RSA were enrolled under double blind randomized trial by randomization in groups of four using a computer-generated list of random numbers. This trial was carried out in which neither the patient, the treating doctors, nor the staff in Medical Genetics of SGPGIMS, Lucknow, with whom the patients dealt, were aware of the source of the therapy the patient received. To keep it blind, blood was collected from both the male and female partner of RSA couples in all cases. A successful pregnancy was considered to be a live birth.

2.2. Immunization protocol

Twenty milliliters of peripheral blood was obtained from each member of the RSA couples and unrelated males (third party) on the basis of prerandomization. Peripheral mononuclear cells were separated aseptically on a Ficoll–Hypaque gradient and then incubated overnight at 37 °C and 5% CO_2 atmosphere. Both the T and B cells were purified from peripheral mononuclear cells by using nylon wool columns, and were used for immunotherapy in women with RSA. For this purpose, each 5×10^6 autologous lymphocyte (AL), third party lymphocyte (TPL), or PL was finally suspended in 1 ml of sterile normal saline (NS), which was then equally distributed into four doses of 0.25 ml each. Each dose (0.25 ml) of cell suspension and neat sterile NS was injected through intradermal (i.d.), intramuscular (i.m.), subcutaneous (s.c.) and intravenous (i.v.) routes at four separate sites on the forearms of women with RSA under medical supervision. Immunization was repeat-

ed at four weekly intervals up to a maximum of six times, and was stopped when MLR-Bf titer of ≥ 30 was achieved. Immunized women with RSA were evaluated for the induction of MLR-Bf development after the completion of immunization course and also during each trimester (12th, 24th and 36th weeks) of pregnancy. The male partner was tested for Rhesus factor (Rh), hepatitis B surface antigen (HbsAg) and human immunodeficiency virus (HIV) antibodies.

2.3. Mixed lymphocyte reaction (MLR)

The mixed lymphocyte reaction blocking antibody (MLR-Bf) was investigated by one-way mixed lymphocyte reaction (MLR) with lymphocytes from RSA couples and also against any other unrelated male (TPL). Mixed culturing of gamma (2800 rads) irradiated stimulator lymphocytes of the male partner or unrelated male (TPL) and responder lymphocytes of the women with RSA, was performed in round-bottomed 96-well plates. Mixed culture was performed by adding 1:1 ratio of each 1×10^5 cells of stimulator and responder lymphocytes in RPMI 1640 which contained either pooled human AB serum or tested serum in each triplicate. Plates were maintained at 37 °C and 5% CO_2 atmosphere. Proliferation was measured at day 6. The cultured cells were harvested on to a glass-fiber filter after a pulse time of 18 h with thymidine(H^3). DNA synthesis was measured by liquid scintillation counting. The percentage (%) inhibition for evaluating the blocking effect of MLR-Bf was calculated by the given formula. When the percentage inhibition was >30 it was considered as MLR-Bf positive

$$\text{Percentage (\%) inhibition} = \frac{\text{mean CPM in control serum} - \text{mean CPM in test serum}}{\text{mean CPM in control serum}} \times 100$$

where CPM = count per minute.

2.4. Statistical analysis

Baseline differences between the treatment and control groups were analyzed by means of analysis of variance (ANOVA), whereas chi-square analysis and *P*-value were corrected with the Bonferroni methods. The strength of the association was estimated by calculating relative risk (RR) by the method of Woolf and Haldane. The *P*-value < 0.001 was considered as statistically significant.

3. Results

A total of 124 women with unknown causes of RSA were registered under double blind randomized trial. Development of MLR-Bf was taken as immunopotentiating factor. Once these antibodies developed, women were advised to conceive. These women with RSA were divided into five groups. In group 1, 28 women received AL. In control group 2, 31 women received immunotherapy with TPL. In group 3, 32 women with RSA received immunization with PL. In group 4, 19 women received treatment with sterile NS. Fourteen women who were found to be positive for MLR-Bf before receiving any therapy were not enrolled for any further treatment (NT) and were considered as group 5. Groups 1, 2, 4 and 5 were considered as control groups, whereas only group 3 was considered as the study group. All of 110 women who received AL, TPL, NS and PL were advised to be followed-up for pregnancy outcome. We also made a record of the natural pregnancy outcome in the remaining 14 women with RSA who were found to be positive for MLR-Bf before receiving any kind of therapy. The mean age and mean number of abortions (Table 1) in women with RSA of groups 1–5 were not significantly different ($P>0.05$). All the women of groups 1, 2, 4 and 5 (92) who received either AL (28), TPL (31), NS (19) or no therapy at all (14) failed to develop a significant level

Table 1
Demographic profile of different groups of women with RSA who participated in double blind randomized trial of paternal lymphocyte immunotherapy

Different groups of women with RSA	Different therapies	Number of women with RSA in each group	Age in years (mean)	Number of abortions (mean)
1	AL	28	26.2 + 2.23	3.6 + 0.21
2	TPL	31	27.6 + 3.26	3.3 + 0.17
3	PL	32	25.92 + 0.65	3.4 + 0.32
4	NS	19	25.13 + 2.67	3.2 + 0.18
5	NT	14	27.96 + 3.89	3.6 + 0.27

AL = autologous lymphocytes, TPL = third party or unrelated male lymphocytes, NS = sterile normal saline, NT = not received any treatment or dropout patients, PL = paternal lymphocytes.

Table 2
MLR-Bf status and pregnancy outcome in paternal lymphocyte-immunized women with RSA under double blind randomized trial

RSA patients no.	Nature, dose and Ix protocol	Percentage inhibition (MLR-Bf)					Pregnancy outcome status
		Before Ix	After Six Ix	During pregnancy			
				12 wks	24 wks	36 wks	
1	5×10^6 PL, 1/4 i.m., 1/4 i.d., 1/4 s.c., 1/4 i.v., 6 identical, Ix was given at 4-week intervals	17.30	82.27	80.83	63.54	36.60	M (3440G)
2	5×10^6 PL, 1/4 i.m., 1/4 i.d., 1/4 s.c., 1/4 i.v., 6 identical, Ix was given at 4-week intervals	12.66	69.35				A (7 weeks)
3	5×10^6 PL, 1/4 i.m., 1/4 i.d., 1/4 s.c., 1/4 i.v., 6 identical, Ix was given at 4-week intervals	14.80	82.34	76.67	54.55	32.00	F (3281G)
4	5×10^6 PL, 1/4 i.m., 1/4 i.d., 1/4 s.c., 1/4 i.v., 6 identical, Ix was given at 4-week intervals	21.75	75.39	72.00	25.00	24.67	M (2422G)
5	5×10^6 PL, 1/4 i.m., 1/4 i.d., 1/4 s.c., 1/4 i.v., 6 identical, Ix was given at 4-week intervals	22.16	78.14	82.98	49.96	32.00	F (3477G)
6	5×10^6 PL, 1/4 i.m., 1/4 i.d., 1/4 s.c., 1/4 i.v., 6 identical, Ix was given at 4-week intervals	15.87	89.16	66.63	51.26	40.87	Nil
7	5×10^6 PL, 1/4 i.m., 1/4 i.d., 1/4 s.c., 1/4 i.v., 6 identical, Ix was given at 4-week intervals	17.35	83.56	77.96	27.00	39.40	M (3120G)
8	5×10^6 PL, 1/4 i.m., 1/4 i.d., 1/4 s.c., 1/4 i.v., 6 identical, Ix was given at 4-week intervals	8.72	68.42	87.98	44.00	42.00	Nil
9	5×10^6 PL, 1/4 i.m., 1/4 i.d., 1/4 s.c., 1/4 i.v., 6 identical, Ix was given at 4-week intervals	7.37	88.60	75.59	58.38	34.07	F (2330G)
10	5×10^6 PL, 1/4 i.m., 1/4 i.d., 1/4 s.c., 1/4 i.v., 6 identical, Ix was given at 4-week intervals	9.61	86.28	83.89	71.03	45.64	M (2324G)
11	5×10^6 PL, 1/4 i.m., 1/4 i.d., 1/4 s.c., 1/4 i.v., 6 identical, Ix was given at 4-week intervals	9.41	51.41	87.94			A (18 weeks)
12	5×10^6 PL, 1/4 i.m., 1/4 i.d., 1/4 s.c., 1/4 i.v., 6 identical, Ix was given at 4-week intervals	10.02	89.41	71.24	57.74	26.94	F (2988G)
13	5×10^6 PL, 1/4 i.m., 1/4 i.d., 1/4 s.c., 1/4 i.v., 6 identical, Ix was given at 4-week intervals	12.51	30.23	80.63	68.20	32.39	M (3368)
14	5×10^6 PL, 1/4 i.m., 1/4 i.d., 1/4 s.c., 1/4 i.v., 6 identical, Ix was given at 4-week intervals	15.00	75.80	74.00	45.39	31.78	F (3240G)
15	5×10^6 PL, 1/4 i.m., 1/4 i.d., 1/4 s.c., 1/4 i.v., 6 identical, Ix was given at 4-week intervals	10.25	72.00	82.67			A (14 weeks)
16	5×10^6 PL, 1/4 i.m., 1/4 i.d., 1/4 s.c., 1/4 i.v., 6 identical, Ix was given at 4-week intervals	13.61	61.40	73.22	67.00	41.75	F (3431G)
17	5×10^6 PL, 1/4 i.m., 1/4 i.d., 1/4 s.c., 1/4 i.v., 6 identical, Ix was given at 4-week intervals	11.90	69.06	55.00	63.74	32.60	Nil
18	5×10^6 PL, 1/4 i.m., 1/4 i.d., 1/4 s.c., 1/4 i.v., 6 identical, Ix was given at 4-week intervals	14.08	69.91	88.89	25.43	29.66	M (2934G)
19	5×10^6 PL, 1/4 i.m., 1/4 i.d., 1/4 s.c., 1/4 i.v., 6 identical, Ix was given at 4-week intervals	23.68	53.61	62.36	45.66	32.74	F (2810G)
20	5×10^6 PL, 1/4 i.m., 1/4 i.d., 1/4 s.c., 1/4 i.v., 6 identical, Ix was given at 4-week intervals	19.59	55.78	23.95	63.89	44.67	F (2720G)
21	5×10^6 PL, 1/4 i.m., 1/4 i.d., 1/4 s.c., 1/4 i.v., 6 identical, Ix was given at 4-week intervals	15.54	62.05	79.40	75.93	22.98	M (3679G)
22	5×10^6 PL, 1/4 i.m., 1/4 i.d., 1/4 s.c., 1/4 i.v., 6 identical, Ix was given at 4-week intervals	26.56	65.50	55.55	54.44	34.74	Nil
23	5×10^6 PL, 1/4 i.m., 1/4 i.d., 1/4 s.c., 1/4 i.v., 6 identical, Ix was given at 4-week intervals	14.55	70.69	60.00			A (18 weeks)
24	5×10^6 PL, 1/4 i.m., 1/4 i.d., 1/4 s.c., 1/4 i.v., 6 identical, Ix was given at 4-week intervals	13.24	70.70	66.33	49.99	55.30	Nil
25	5×10^6 PL, 1/4 i.m., 1/4 i.d., 1/4 s.c., 1/4 i.v., 6 identical, Ix was given at 4-week intervals	16.83	64.40	88.84	67.26	31.06	F (3660G)

Table 2 (*continued*)

RSA patients no.	Nature, dose and Ix protocol	Percentage inhibition (MLR-Bf)					Pregnancy outcome status
		Before Ix	After Six Ix	During pregnancy			
				12 wks	24 wks	36 wks	
26	5×10^6 PL, 1/4 i.m., 1/4 i.d., 1/4 s.c., 1/4 i.v., 6 identical, Ix was given at 4-week intervals	10.02	59.80	42.36	45.78	23.82	M (2983G)
27	5×10^6 PL, 1/4 i.m., 1/4 i.d., 1/4 s.c., 1/4 i.v., 6 identical, Ix was given at 4-week intervals	20.00	51.53	35.67	58.49	33.33	F (3264G)
28	5×10^6 PL, 1/4 i.m., 1/4 i.d., 1/4 s.c., 1/4 i.v., 6 identical, Ix was given at 4-week intervals	18.56	63.44	78.93	34.78	30.84	Nil
29	5×10^6 PL, 1/4 i.m., 1/4 i.d., 1/4 s.c., 1/4 i.v., 6 identical, Ix was given at 4-week intervals	17.69	59.41	23.94	61.20	41.67	F (3635G)
30	5×10^6 PL, 1/4 i.m., 1/4 i.d., 1/4 s.c., 1/4 i.v., 6 identical, Ix was given at 4-week intervals	15.29	51.84	77.77	48.06	38.95	M (3177G)
31	5×10^6 PL, 1/4 i.m., 1/4 i.d., 1/4 s.c., 1/4 i.v., 6 identical, Ix was given at 4-week intervals	16.83	31.14	68.35	42.56	32.00	Nil
32	5×10^6 PL, 1/4 i.m., 1/4 i.d., 1/4 s.c., 1/4 i.v., 6 identical, Ix was given at 4-week intervals	18.47	36.26	92.64	72.66	62.93	M (3228G)

PL = paternal lymphocytes, A = abortion, F = female, M = male, Ix = immunizations, G = weight in grams, wks = gestational age in weeks.

(>30) of MLR-Bf. However, 48 of these 92 women became pregnant, but only 16 (33%) gave birth to normal healthy children and 32 (67%) were aborted again. However, 25 of 32 women with RSA of group 3, who received immunization with PL, showed a significantly increased level of MLR-Bf (>30) and gave birth

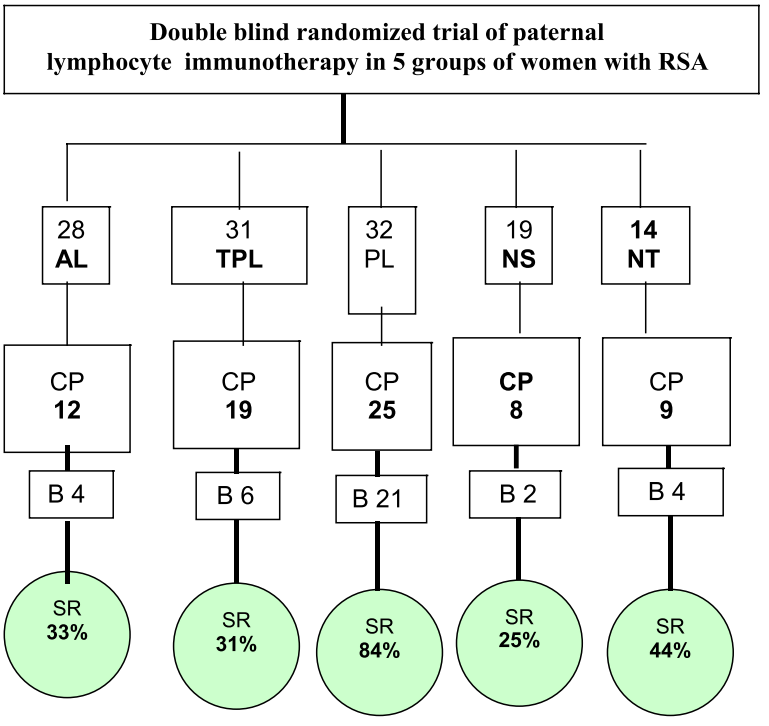

Fig. 1. AL = autologous lymphocytes, TPL = third party or unrelated male lymphocytes, NS = sterile normal saline, NT = not received any treatment or dropout patients, PL = paternal lymphocytes, CP = confirmed pregnancy, B = live birth, SR = success rate in percentage (%).

Table 3
Double blind randomized trial of paternal lymphocyte immunotherapy for women with RSA

Different groups of women with RSA	Different therapies	Number of women with RSA in each group	Confirmed pregnancy	Live births	Abortion	Success (%)
1	AL	28	12	4	8	33
2	TPL	31	19	6	13	31
3	PL	32	25	21	4	84
4	NS	19	8	2	6	25
5	NT	14	9	4	5	44

AL = autologous lymphocytes, TPL = third party or unrelated male lymphocytes, NS = sterile normal saline, NT = not received any treatment or dropout patients, PL = paternal lymphocyte, RSA = recurrent spontaneous abortion, MLR-Bf = mixed lymphocytes reaction blocking antibodies.

to normal healthy babies (84%), whereas the remaining 7 women with RSA who failed to develop appropriate level of MLR-Bf (< 30), aborted again (Table 2). Thus, overall success rate of immunotherapy with PL was 84% as compared to AL (33%), TPL (31%), NS (25%), and NT (44%; Fig. 1 and Table 3). In this study, we have not seen any adverse effects of immunotherapy as we have carried out follow-up for 1.5 years for all the successful pregnancies.

4. Discussion

In this study, we examined the MLR-Bf development and its correlation with the successful pregnancy outcome in women with RSA when they were treated either with PL or with other therapies (AL, TPL, NS and NT). Various studies demonstrated that immunization with PL attributed to the success of pregnancy in women with RSA [21,24,31,45–47]. In the present study, we have registered 124 women with RSA for immunotherapy and compared the effect of PL immunotherapy in different groups of women with RSA by using double blind randomized trials. Our results revealed that 84% of PL-immunized women with RSA showed a significantly increased level of MLR-Bf development, which was directly correlated, with the successful outcome of pregnancy. However, other control group women, who received treatment with AL (28), TPL (31), sterile NS (19) or no treatment at all (14), showed a significantly decreased level of MLR-Bf development and subsequently, 33%, 31%, 25% and 44% successful pregnancy outcome. Upon comparison of various updated randomized trials of PL immunotherapy for women with RSA [29,32,40,42,49–55], we found a significant higher pregnancy outcome (62%) among the women of the study group who received immunization with PL as compared to women of the control group (52%), who received immunization either with AL, TPL, NS or no treatment at all (NT). This difference was statistically significant ($P < 0.01$; Table 4). However, Ober et al. [55] reported that this mode of therapeutic approach does not improve pregnancy outcome in women with RSA. But they did not analyze the results in the light of previous miscarriage numbers. They considered the cases which did not achieve pregnancy in 12 months after immunotherapy as failures. Our study and most of the other studies analyzed only the successful outcome. They used PL which were stored overnight, rather than fresh cells, and did not fully exclude patients with autoimmunity. Stored cells might lose immunogenic effects, and certain types of patients with autoimmunity, such as antiphospholipid antibody, antinuclear antibody, antithyroid antibody and antiendothelial cell antibody may not respond well. The discrepancy between their data and ours is probably because they did not use any criteria, such as APCA, Ab2 and MLR-Bf, for patient selection for immunotherapy. The results of the present study showed that women with RSA of negative MLR-Bf group were found to have significantly benefited from PL immunotherapy. On the other hand, 9 of the 14 women having RSA with positive MLR-Bf who did not receive any kind of therapy became pregnant. Of these 9 women, 4 who showed an increased level of MLR-Bf development during the first, second and third trimesters delivered healthy outcomes, whereas the remaining 5 who showed a decreased level of MLR-Bf development throughout the gestation experienced fetal losses. The data of immunotherapy during early pregnancy should be analyzed in the light of the number of previous miscarriage and gestational window. Most of the controversial studies performed immunotherapy only once or twice before pregnancy [50,55], whereas in most of the successful studies, PL immunization was performed either at the regular interval

Table 4
Comparison of the results of randomized trials of paternal lymphocytes immunotherapy for women with RSA

Randomized trials	Study group (%)	Control group (%)	Immunization protocol
Carp et al. [54]	5/11 (45)	11/31 (35)	SIx: $80-100 \times 10^6$ PL from 100 ml blood of male partner, 2/3 i.v., 1/6 i.d., 1/6 s.c., immunizations were repeated at 3–4 week intervals until APCA became apparent in the cross-match
Cauchi et al. [50]	13/21 (62)	19/25 (76)	SIx: $10-100 \times 10^6$ PL from 150 ml of blood of male partner, 1/2 i.v., 1/4 i.d., 1/4 s.c. CIx: 2 ml nornal saline, 1/2 i.v., 1/4 i.d., 1/4 s.c.
Clark and Daya [49]	7/11 (64)	2/7 (29)	SIx: 40×10^6 PL from blood of male partner, s.c. CIx: saline, s.c.
Daya and Gunby [40]	91/136 (66)	60/119 (50)	SIx: $50-60 \times 10^6$ PL from male partner, sc. CIx: saline
Gatenby et al. [52]	13/19 (68)	9/19 (47)	SIx: 400×10^6 PL from 400 ml of blood of male partner, 2/3 i.v., 1/3 i.d., s.c. CIx: 400×10^6 AL, 2/3 i.v., 1/3 i.d., s.c.
Ho et al. [51]	33/39 (84)	39/60 (65)	SIx: $100-200 \times 10^6$ PL/TPL, i.d., boost with 50 ml of blood mononuclear cells at 6 months of gestation if not pregnant CIx: $100-200 \times 10^6$, AL from women with RSA, i.d.
Illeni et al. [53]	10/22 (45)	11/22 (50)	SIx: 200×10^6 PL from 400 ml blood of male partner, 1/3 i.v., 1/3 i.d., 1/3 s.c. CIx: nil
Li et al. [42]	17/20 (86)	17/22 (86)	SIx: PL from 10 ml blood of male partner, .i.v., three times or more at an interval of 4 weeks CIx: TPL, from 10 ml blood of other than male partner, i.v., three times or more at an interval of 4 weeks
Mowbray et al. [32]	17/22 (77)	10/27 (37)	SIx: $100-900 \times 10^6$ PL from 1 unit of blood 2/3 i.v., 1/6 i.d., 1/6 s.c. CIx: $30-80 \times 10^6$ AL from 40 ml of autologous blood, 2/3 i.v., 1/6 i.d., 1/6 s.c.
Ober et al. [55]	31/86 (36)	41/85 (48)	SIx: 200×10^6, PL from 1 U blood of male partner, 2/3 i.v., 1/6 i.d., 1/6 s.c. CIx: 5 ml normal saline administered in an identical manner
Regan et al. [29]	34/46 (74)	0/4 (0)	SIx: PL from 400 ml blood of male partner, i.v., two boosters were given at <6 weeks of gestation CIx: AL from 400 ml blood of women with RSA, two boosters were given at <6 weeks of gestation
Total	271/433 (62)	219/421 (52)	

PL = paternal lymphocytes, AL = autologous lymphocytes, TPL = third party lymphocytes, NS = normal saline, NT = not received any treatment, i.v. = intravenous immunization, i.d. = intradermal immunization, i.m. = intramuscular immunization, i.c. = intracutaneous immunization, s.c. = subcutaneous immunization, SIx = immunization in study group, CIx = immunization in control group, U = unit, wks = weeks. Randomized trial for paternal lymphocyte immunotherapy in women with RSA ($P < 0.01$).

of 3–6 weeks before pregnancy [31,45] or once before, following the course of immunization, and a few more immunizations during pregnancy [56].

Various risk and side effects (twin pregnancies, preterm delivery, growth retardation, neonatal thrombocytopenia and certain congenital abnormalities) have been reported to be involved in PL-immunized women with RSA [49,55,57–61]. However, we could not find any side effects in PL-immunized women with RSA throughout the 1.5 years of their continuous follow-up.

The exact mechanism of PL immunization in women with RSA is still not clear, However, it has been reported that expression of APCA [22], Ab2 [21], MLR-Bf [31,45] and progesterone-induced blocking factor (PIBf) [62] in PL-immunized women with RSA was associated with successful pregnancy. These factors may protect the fetus from the toxic effect of the mother's immune system and make the pregnancy successful by balancing the Th-2 shift and inhibition in overactivity of NK cells [27]. Some of the recent studies suggested that PL immunization is also correlated with the modulation of immunity in women with unexplained RSA as it reduced the NK cell activity and performed the shift from Th1-type reactivity to Th2-type reactivity [47,48]. However, the exact mechanisms by which MLR-Bf make favourable environment for the success of pregnancy and

protection of fetal loss is still not clear, but these are now under examination.

5. Conclusion

Results of the present study suggested that PL-immunized women with RSA showed a significant high level of MLR-Bf and pregnancy outcome compared to women with RSA who received AL, TPL, NS or no treatment at all.

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