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REVIEW ARTICLE

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Lymphocyte immunotherapy and its probable mechanism in the maintenance of pregnancy in women with recurrent spontaneous abortion

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Abstract *Introduction:* Most women with alloimmune cause of recurrent spontaneous abortion (RSA) includes increased sharing of human leukocyte antigens (HLA) that may prohibit the mother from making anti-paternal cyto-toxic antibodies (APCA), anti-idiotypic antibodies (Ab2) and mixed lymphocyte reaction blocking antibodies (MLR-Bf). Overactivity of T helper-1 (Th-1) cytokines and natural killer (NK) cells have been also reported to be the major alloimmune cause of recurrent spontaneous abortion (RSA). It was revealed from extensive updated analysis of this subject that paternal lymphocytes immunotherapy may play a significant role in the prevention of alloimmune cause of fetal loss in women with RSA. These alloimmune parameters are found to be suppressed in successful immunotherapy, which is comparable to normal pregnancy. *Review and discussion:* Various studies represented that paternal lymphocyte immunotherapy was attributed to the high expression of APCA, Ab2, MLR-Bf and inhibition of Th-1 pattern of cytokines and NK cell activity in women with alloimmune cause of RSA. Present updated randomized clinical trials demonstrated that women with RSA of study group who have been treated with paternal lymphocyte immunotherapy had more successful outcomes (68%) as compared to women with RSA of control group who either received autologous lymphocytes/third party lymphocytes/normal saline or no therapy (54%), ($p<0.02$). However, when the results of the randomized and nonrandomized studies were pooled together it was observed that 67% of women with RSA of study group who received paternal lymphocyte immunotherapy showed successful pregnancy outcome in comparison to 36% success in women with RSA of control group who either received autologous lymphocytes/third party lymphocytes/normal saline or no therapy ($p<0.05$). *Conclusion:* These results advocate the role of paternal lymphocyte immunotherapy for the maintenance of pregnancy in women with RSA.

Keywords Recurrent spontaneous abortion · Antipaternal cytotoxic antibody · Anti-idiotypic antibodies · Mixed lymphocyte reaction blocking factor · Immunoglobulin-3 · Immunotherapy

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Introduction

It has been reported that recurrent miscarriage is the commonest complication of pregnancy affecting approximately 1 in 300 pregnant women [100, 118]. Recurrent spontaneous abortion (RSA) can be defined as occurrence of three or more clinically detectable pregnancy failure before the 20th weeks of gestation from the last menstrual period or less than 500 g of fetal body weight [21, 29, 105]. 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 [78, 103], anatomical [43, 102, 120], endocrine [19], placental anomalies [49, 62, 116], smoking and alcohol consumption [37], exposure to environmental factors such as lead, mercury, ethylene oxide and ionizing radiations [89], and to immunological factors [1, 2, 3, 48, 56, 67, 79, 83, 87, 92, 112]. 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 suggest 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 [95, 117].

The immunological relationship between the mother and the fetus is a bidirectional communication determined on one hand by fetal antigen presentation and on other hand by recognition and reaction to these antigens by the maternal immune system. There are evidences that reveal 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 [22, 93]. 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 anti-paternal cyto-toxic antibodies (APCA), anti-idiotypic antibodies (Ab2) and mixed lymphocyte reaction blocking antibodies (MLR-Bf). Immunotherapy with paternal lymphocytes is an effective treatment for unexplained recurrent spontaneous abortions as it was attributed to the production of APCA [1, 65, 75, 79, 96], Ab2 [48, 65, 80, 106] and MLR-Bf [1, 2, 3, 9, 26, 63, 68, 75, 83, 90, 93, 100, 110, 111, 112, 125] during pregnancy in women with RSA. These antibodies may play an important role in the maintenance of pregnancy. Where as several studies have shown that the absence or low expression of APCA, Ab2 and MLR-Bf may cause recurrent fetal loss in women with RSA [2, 3, 65, 68, 80, 83, 93].

Several studies have reported that activation of the maternal NK cells induces subsequent abortion in women with normal chromosomes. The proposed mechanism is that maternal NK cells activate Th1 cells for secretion of cytokines that are toxic to the trophoblast [17, 27, 55, 93, 122]. Hence it is possible that suppression of NK cell activity may help in the maintenance of the fetal allograft during pregnancy. However, decidual NK cells are not cytolytic, but produce IFNγ which activates the decidual macrophage (MØ) for the production of high levels of nitric oxide (NO) and tumor necrosis factor-alfa (TNFα) which damage to the conceptus not by direct lysis of trophoblast cells but through apoptosis and causes the inhibition of secretion of granulocyte macrophage colony stimulating factors (GM-CSF) from the uterine epithelium [35, 119, 121, 123]. MØ also produces IL-12 in response to certain microbial products (LPS, IFNγ) during infectious diseases, which stimulates NK cells and cytotoxic T cells (CTLs) to secrete IFNγ that enhances Th1 development and induces the secretion of tumor necrosis factor-alfa (TNF-α), TNF-beta (TNF-β) and interleukin-2 (IL-2). These Th-1 cytokines may proposed to stimulate pregnancy failure via embryo and trophoblast toxicity [5, 18, 42, 43, 45, 64, 88, 91, 92]. Whereas Th2 cells produce IL-4, IL-5, IL-10 and IL-13 which promote success of the pregnancy by counter inflammation and suppression of the NK cell activity [17, 18, 20, 36, 38, 40, 43, 44, 59, 91, 98, 99]. The predominance of a given cytokines in the microenvironment at the time of antigen presentation is an important factor in driving naive $CD4^+$T cells toward Th-1 or Th-2 dominated population. In some of the recent studies it has been demonstrated that paternal lymphocyte immunotherapy caused significant inhibition in the level of Th-1 cytokines and NK cells activity [32, 39, 61].

Mechanism of paternal lymphocyte immunotherapy in women with RSA

The exact mechanisms of immunotherapy against paternal lymphocytes have yet to be elucidated. The initial reports on paternal lymphocyte immunization used the sharing of HLA antigens between spouses as a criteria for immunization [9, 115]. This excess sharing was considered to be increased in couples with RSA [56] and responsible for the hyporesponsiveness was considered to be shown by a lower incidence of APCA, Ab2 and MLR-Bf in RSA couples [75]. Some investigators suggested that paternal lymphocyte immunotherapy may act as immunogen to enhance the maternal immune response and to induce various humoral antibodies as an immunological regulators for maintaining pregnancy. It was further suggested that humoral antibodies (APCA, Ab2 and MLR-Bf) produced as a result of immunotherapy [83] would mask the fetal HLA antigens and prevent them from being attacked by the maternal T cells (Fig. 1). We have recently demonstrated that MLR-Bf developed as a result of lymphocyte immunotherapy was IgG3 in nature and associated with success of pregnancy in women with RSA.

The anti T cell receptor (TCR) idiotypic antibody that has been reported to be present in the sera of normal pregnant women [48] would provide another mechanism for the immunotherapy. After immunization with paternal lymphocytes, maternal T cells recognizing paternal HLA antigens (one of the HLA antigens of the fetus) would expand and serve as immunogens to produce anti TCR-idiotypic antibodies. The anti TCR idiotypic antibody would then bind specifically to the TCR and suppress the maternal immune response against the fetus, allowing the fetus to escape the maternal immunological attack. Thus the beneficial effect of this procedure has been attributed to the induction of various humoral antibodies that may block the mechanism of immune-rejection of the fetus and help in the implantation and fetal growth [48, 63, 68, 79, 90, 93, 125]. The beneficial effect of paternal lymphocyte immunotherapy also include specific and non specific T cell suppression [10, 71], a decrease in the level of maternal IL-2 receptors [53], which shifts Th1 to Th2 type immunity [39] and causes decrease in NK cell activity [61]. Various recent Th1/Th2 theories may support these beneficial effects to maintain pregnancy [40, 44, 64, 91, 92].

Recently Gafter et al. [32] demonstrated the culture of monocytes of alloimmunized women with RSA and found that paternal lymphocyte immunotherapy causes the reduction in level of IL-6. There were no previous reports about the serum levels of this pleiotropic cytokine and its receptor in women with RSA. The result obtained through this work showed that there are highly significant difference in the IL-6 and its soluble receptor levels

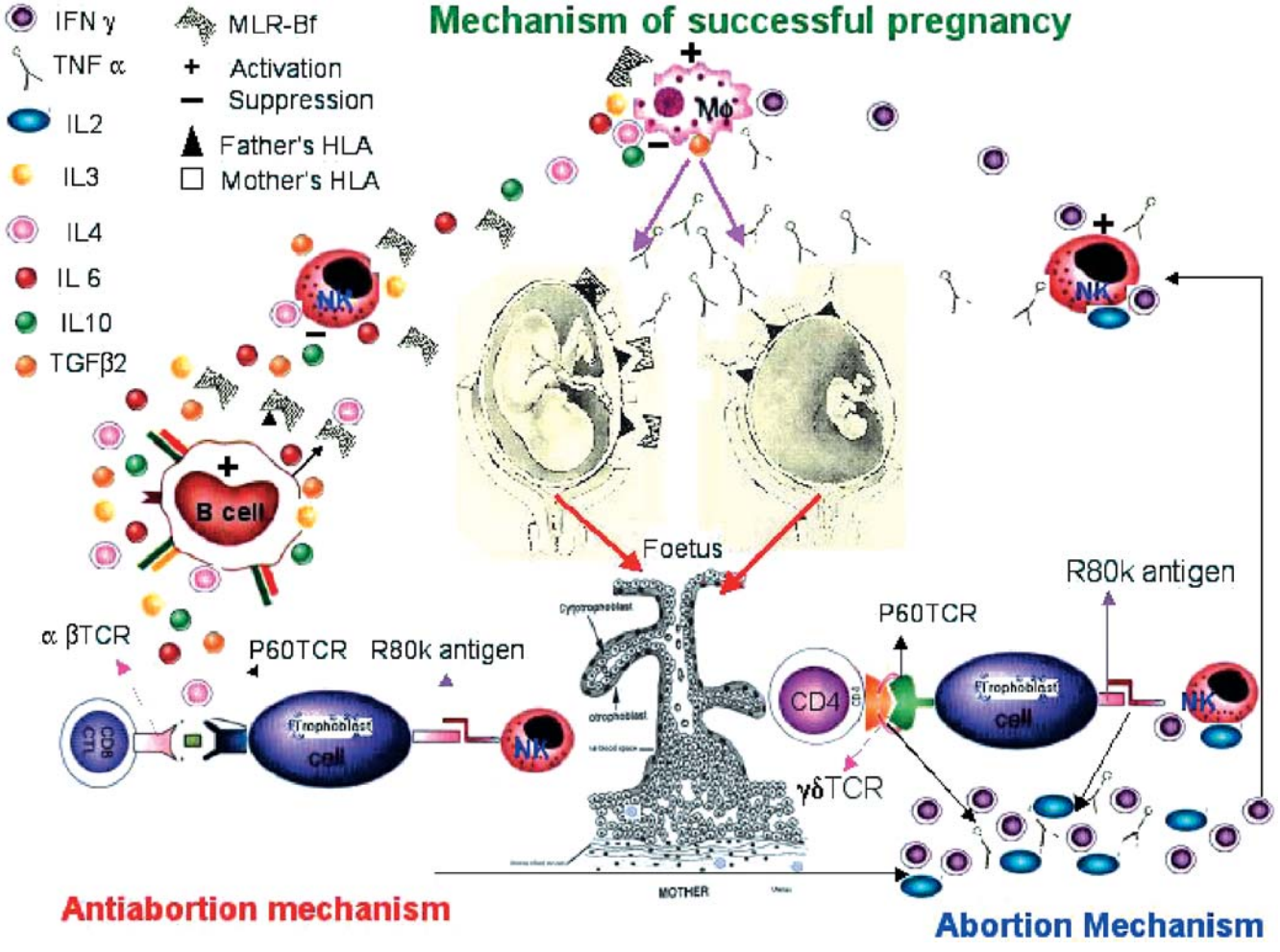

Fig. 1 Role of MLR-Bf in the maintenance of pregnancy. Trophoblast bears the P60 receptor and R80 K (80 kDa) antigens on its surface. P60 receptors when binds with the $\gamma\delta$ T cell receptor of CD4 cells and R80 K antigen when recognized by the $\gamma\delta$ T cell receptor of NK cells , Th1 patterns of cytokines (IFN γ, TNFα, and IL-2) are produced which kill the fetal cells). On the contrary $\alpha\beta$ T cell receptor of $CD8^+$ cells and P60 T cell receptor of trophoblast signals for secretion of Th2 pattern of cytokines (IL-4, IL-5, IL-10, IL-13) and MLR-Bf that inhibits NK activity and the activity of TNF α producing macrophages and protects the fetal cells by lysis caused by host effector cells

between the women with RSA that had received and those who had not received lymphocyte immunotherapy. Some of the in vitro studies demonstrated that low dose of IL-6 stimulate the asymmetric antibodies synthesis and a high dose decreases it. Thus it would be suggested that IL-6 regulate the synthesis of asymmetric antibodies in the trophoblast that block the placental antigen (R80K) [124] and NK cells [50]. HLA-G antigens expressed on trophoblast cells are reported to inhibit NK cell activity [30, 58, 69, 84, 97] during normal pregnancy. However, increased number of NK cells has been observed in women with RSA [6]. Embryo rejection in animal models also appears to depend upon activated natural killer (NK) cells rather than on antigen specific effector cells. Alternatively these proinflammatory cytokines may convert NK cells to lymphokine activated killer (LAK) cells, which have been shown to lyse trophoblast cells. High systemic levels of LAK cells may correlates with the high abortion rates. Paternal lymphocyte immunotherapy seems to develop anti-R80K asymmetric antibodies, which suppress the activity of NK cell and prevent the fetal loss in women with RSA.

It was further reported that trophoblast bears P60 receptor and R80 K (80 kDa) antigens on its surface. P60 receptors when binds to the gamma delta T ($\gamma\delta$ T) cell receptor of CD4 cells and R80 K antigen gets recognized by the $\gamma\delta$ T cell receptor of NK cells, Th1 patterns of cytokines are produced which kill the fetal cells [5, 119]. NK-$\gamma\delta$ T cells and $\gamma\delta$ T cells have been implicated in fetal resorptions and elimination of these NK-T cells prevents abortions. On the contrary, when alpha beta-T ($\alpha\beta$-T) cell receptor of $CD8^+$ cells and P60 T cell receptor of trophoblast cells bind with each other, $CD8^+$ cells may become $CD4^-$ $CD8^-$. This modified $CD8^+$cells has anti-inflammatory properties and cause the change in the signal for secretion of Th2 pattern of cytokines (Fig. 1).

Arck et al. [5] points out that these populations have different and opposite effects when activated, the NK $\gamma\delta$ T cell is more Th1-like as they produce IFNγ, TNFα and IL-2, while the $\gamma\delta$ T cell is more Th2-like as it secretes IL-10 and TGFβ2.

It has been explained that the failure of anti CD4 and anti CD8 antibodies decrease the abortion rates in mice with recurrent spontaneous abortion [23, 31]. Injection of anti $\gamma\delta$ TCR antibodies, anti-IFNγ antibodies and anti-NK antibodies into pregnant mice may decrease abortion rates. Lymphocyte immunization in women with RSA seems to cause transformation of $CD8^+$ $\alpha\beta$ cells to $CD4^-$ $CD\ 8^-$ cells as well as independent production of IL-4 and IL-10, which protects the abortion by inhibition of NK cell activation, counter inflammation and production of alloantibody formation [44, 59, 99]. Check et al. [15] was reported that lymphocyte immunization causes an increase in progesterone induced blocking factor (PIBF) in women with RSA, which may play a significant role in the maintenance of pregnancy by regulating the Th2 shift. This shift induces some T cells that utilize different TCR repertoires and possibly suppresses maternal rejection reactions and causes the maintenance and continuation of successful pregnancies [38, 39, 107]. Same effect was also predicted when paternal lymphocytes immunization induced APCA, Ab2 and MLR-Bf in women with RSA. An increased level of peripheral blood $CD\ 56^+$ $CD3^-$ NK cell levels and NK cell cytotoxicity against K562 [41, 60] cells and significant decrease in these parameters [61, 108] has been noticed in alloimmunized women with RSA. In most women who experience recurrent miscarriage and no cause can be identified, the humoral and cellular immune mechanisms may be responsible which prevent mothers from developing protective immune responses which are essential for the survival of the semiallogeneic pregnancy. However, failure of these immune mechanisms has been proposed as a cause for 50% of all these losses. Thus the mechanism of this therapeutic effect in alloimmune pregnancy loss concerns with the expression of APCA, Ab2 and MLR-Bf as well as inhibition in the induction of Th1 cytokine and NK cell activity during pregnancy in women with RSA.

Various clinical trials of lymphocyte immunotherapy using for women with RSA

Taylor and Faulk [115] first reported paternal lymphocyte immunotherapy for women with unexplained RSA. Since then it has become widely performed with various reports of its efficacy and safety. They originally based their idea of third party lymphocyte immunization for women with RSA on observations that renal allograft rejection could be delayed by third party blood transfusions while Beer et al. [9] based paternal lymphocyte immunization on their belief that maternal "blocking antibodies" were necessary for successful pregnancy. Various recent studies demonstrated that humoral antibodies like APCA [60, 72, 79, 108] anti TCR idiotypic antibodies [48] and MLR-Bf [2, 3, 68, 83, 93] were produced during pregnancy as well as after paternal lymphocyte immunotherapy in women with RSA were correlated with the success of pregnancy. In addition, other beneficial effect of this therapeutic approach was found to be associated with non specific T cell suppression [10, 71], a decrease in the level of maternal IL-2 receptors [53], shift to Th2 type immunity [32, 39] and suppression of NK cell activity [32, 61]. Thus there is popular current belief that whenever there is failure of induction of these protective immune response, failure of pregnancy occurs [16]. Some of the published results of randomized allogenic lymphocyte immunotherapy for women with RSA did not confirm the efficacy of immunotherapy [14, 28, 47, 51, 63, 77]. They suggested that there are some other factors such as the number of previous miscarriages, presence of prior live births, and time of conception after immunization and patient's age which may also influence the outcome of pregnancy [14]. However, a recent study [77] 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 used paternal lymphocytes, which were stored for overnight rather than fresh cells, and did not fully excluded patients with autoimmunity. Stored cells might lose immunogenic effects and certain types of patients with autoimmunity, such as antiphospholipid antibody and antinuclear antibody, may not respond well. They considered the cases that did not achieve pregnancy in 12 months after immunotherapy, as a failure. However, our study and most of the other studies analyzed only the successful outcome. The data of immunotherapy during early pregnancy should be analyzed in the light of number of previous miscarriage and gestational window. Most of the controversial studies performed immunotherapy only once or twice before pregnancy whereas in most of the successful therapies immunization was performed at the regular interval of 2–4 weeks before the pregnancy and again few immunizations during pregnancy.

However, few randomized and large number of nonrandomized trials in which paternal lymphocyte were used for immunotherapy revealed significant obstetric outcomes in women with RSA as compared to control group where no immunotherapy was given or other sources of lymphocytes were used [1, 2, 4, 7, 8, 11, 13, 15, 16, 25, 26, 32, 33, 34, 39, 46, 48, 52, 54, 57, 66, 68, 70, 72, 73, 74, 75, 76, 77, 81, 82, 86, 90, 93, 95, 96, 100, 101, 104, 109, 110, 111, 112, 113, 115]. When we analyzed the randomized trial alone we found that very few women with RSA had complete details available as to whether they seroconverted to any one of humoral antibodies (APCA, Ab2, anti-autologous TCR antibodies, MLR-Bf, PIBF). We did meta analysis of various randomized and nonrandomized clinical trials using paternal lymphocytes for immunotherapy in women with RSA as a study group where autologous lymphocytes, third party lymphocytes, normal saline was received and also the women who did not received any kind of treatment. When we compared the success rate in

randomized trials [14, 16, 26, 28, 34, 46, 47, 51, 63, 75, 77, 82, 95] we found 68% success rate in the study group as compared to a 54% success rate in women with RSA of control group, which was statistically significant ($p<0.02$). When the results of the randomized and nonrandomized studies were pooled (Table 1), the benefit was further seen in paternal lymphocyte immunized women with RSA of study group. However, on comparing the success rate between the pooled data of study and control group of randomized and nonrandomized trials we found 67% success rate in paternal lymphocytes immunized women with RSA under study group as compared to 36% success rate in women with RSA who received autologous lymphocytes or third party lymphocytes or normal saline or who did not received any kind of treatment under control group. This difference was reached at a level of significance ($p<0.05$), which supports and favors the success and efficacy of paternal lymphocyte immunotherapy as a therapeutic approach for the treatment of women with RSA. However, the drawback of these studies is the small sample size and few of these trials have no control groups for comparisons. Women randomized to immunotherapy tended to be older and reported for more spontaneous abortions than those randomized to other treatment (autologous cells, third party cells, saline) or no treatment. Although these differences were not significant, we cannot exclude a potentially worse prognosis in women allocated to immunotherapy since there were older women in this group compared with the control group. It was evident from various randomized and nonrandomized trials that there are three kinds of immunotherapy with the paternal lymphocytes for the treatment of women with RSA:

1. Immunization performed before pregnancy
2. Immunization performed during pregnancy
3. Immunization performed before and during pregnancy

In theory immunotherapy performed before pregnancy may be beneficial for preventing extremely early abortions, because the mother was already immunized when she conceives. Cowchock and Smith [24] reported a better result of immunization early in pregnancy. However, on considering the limited duration of immunotherapy efficacy, the RSA patients have to receive immunotherapy repeatedly until they become pregnant. On the other hand, immunotherapy, if performed after pregnancy is established [4] and may be efficacious in maintaining pregnancy there after but not in preventing extremely early abortions. However Maejima et al. [66] reported that immunotherapy with the paternal lymphocytes performed before and during pregnancy produces a better outcome as compared when it is performed only before pregnancy. While Kilpatrick and Liston [54] reported that 28 women of recurrent miscarriage who had live births after paternal lymphocyte immunization were followed while 16 had subsequent pregnancies without further treatment. One pregnancy was terminated and 5 others were spontaneously aborted. Success rate of immunotherapy was 67%. Additional immunotherapy is not necessary for patients who have obtained successful results after the initial immunotherapy and are positive for MLR-Bf antibodies after their first delivery [111]. Carp et al. [13] reported that the RSA women most likely to benefit from immunotherapy are the primary or secondary aborters in whom the immune parameters may change after immunization. They, however, stressed that booster immunization may be necessary to maintain seroconversion. Tamura et al. [112] reported that titer of MLR-Bf increases with progression of pregnancy. Once these blocking antibodies developed, it is helpful in the subsequent pregnancies. Where as Reznikoff et al. [96] found a positive association between seroconversion for cytotoxic antibodies and gestational success in paternal lymphocyte treated patients. They evaluated 34 patients and found that 27 (93%) of 29 patients who were seroconverted had a live child, whereas 3 (60%) of 5 who did not had a new abortion. Similarly, Carp et al. [12] reported that 27 (72%) of 89 patients who were seroconverted had gestational success and 10 (63%) of 16 of those who were not responding had again shown failure. The result of the international collaborative study and meta analysis on allogenic lymphocyte immunization for women with RSA [26] showed that success is associated with the presence of antipaternal antibodies in both treated and control patients. But, this study does not make it clear whether these antibodies are cytotoxic antibodies or MLR-Bf. However Pena et al. [86] reported that the alloimmunization induced MLR-Bf in women with RSA was not associated with successful out come of pregnancy. They have immunized 33 RSA women with paternal lymphocytes and found that 23 (80%) of 33 women with RSA had a live child, of those women with RSA having success, only 50% produced MLR-BF. Of those patients having a new loss, 5 did and 2 did not produce MLR-Bf while 2 women with RSA who also showed pregnancy failure did not produce MLR-Bf ($p<0.05$). Regarding the 17 patients tested for cytotoxic antibodies 4 of the 5 patients who tested positive had a new abortion, whereas 1 of 12 whose tests remained negative did not show any gestational success.

Our work in this direction to understand the maternal reactivity in RSA was based on a series of studies on 105 women with a history of at least three consecutive unexplained abortions and 60 women 15 each from all the trimesters and 15 from post partum period with a history of at least three successful pregnancies. We have demonstrated that APCA, Ab2 and MLR-Bf developed during pregnancy and after immunotherapy in women with RSA [1, 2, 3, 80]. These antibodies are specific to paternal lymphocytes [2, 3]. We have also characterized MLR-Bf from the sera of normal pregnant and post immunized women with RSA and demonstrated that it was immunoglobulin-3 (IgG 3) in nature [83]. We immunized 73 women with RSA under nonrandomized trial and 32 women with RSA under a double blind randomized trial. A closer examination of the immunotherapy revealed that the success rate of pregnancy in the

Table 1 Comparison of the results of randomized and nonrandomized clinical trials of paternal lymphocytes immunotherapy for women with recurrent spontaneous abortion. *PL* paternal lymphocytes, *AL* autologous lymphocytes, *TPL* third party lymphocytes, *NS* normal saline, *NT* no 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

Reference of study	Study group	Control group	Immunization protocol
[115]	3/4 (75)		SIx: PL from each 1 U blood of 25 donors, i.v., identical immunization was repeated at intervals of about 3 weeks); CIx: nil
[7]	100/121 (83)	13/51 (25)	SIx: 160×10^6 PL, i.d.; CIx: nil
*[75]	17/22 (77)	10/27 (37)	SIx: $100–900\times10^6$ PL from 1 U of blood 2/3 i.v., 1/6 i.d., 1/6 s.c.; CIx: $30–80\times10^6$ AL from 40 ml of autologous blood, 2/3 i.v., 1/6 i.d., 1/6 s.c.
[109]	7/10 (70)		SIx: $50–80\times10^6$ PL from 100 ml blood of male partner, i.d.; CIx: nil
[8]	8/39 (72)	16/44 (36)	SIx: 80×10^6 PL from male partner, i.v., i.d., s.c.; CIx: NT
[76]	5/13 (68)		SIx: $<150\times10^6$ PL from I U blood of male partner, 2/3 i.v., 1/6 i.d., 1/6 s.c.
	19/22 (86)		SIx: $>150\times10^6$ PL from 1 U blood of male partner, 2/3 i.v., 1/6 i.d., 1/6 s.c.
[74]	164/229 (72)		SIx: PL from 20 ml of blood of male partner, 2/3 i.v., 1/6 i.d., 1/6 s.c.; CIx: nil
[96]	28/33 (85)	15/31 (48)	SIx: 200×10^6 PL of male partner i.v., i.d., s.c.; CIx: NT
[101]	27/34 (80)	2/9 (22)	SIx: $>150\times10^6$ PL of male partner, i.v., i.d., s.c.; CIx: NT
*[95]	34/46 (74)	0/4 (0)	SIx: PL from 400 ml blood of male partner, i.v., two booster were given at <6 weeks of gestation; CIx: AL from 400 ml blood of women with RSA, two booster were given at <6 weeks of gestation
[33]	15/28 (55)		SIx: 80×10^6 PL from 50 ml blood of male partner i.d., boost 4–6 weeks later; CIx: nil
[11]	79/156 (50)	11/30 (36)	SIx: $>120\times10^6$ PL of male partner, i.d., s.c.; CIx: NT
[110]	28/35 (80)	4/12 (30)	SIx: 100×10^6 PL from 100 ml of blood of male partner, i.d.; CIx: NT
[25]	43/74 (58)	8/25 (32)	SIx: 150×10^6 PL from 1 U blood of male partner, i.v., s.c.; CIx: NT
*[14]	13/21 (62)	19/25 (76)	SIx: $10–100\times10^6$ PL from 150 ml of blood of male partner, 1/2 i.v., 1/4 i.d., 1/4 s.c.; CIx: 2 ml normal saline, 1/2 i.v., 1/4 i.d., 1/4 s.c.
*[46]	33/39 (84)	39/60 (65)	SIx: $100–200\times10^6$ PL/TPL, i.d., boost with 50 ml of blood mononuclear cells at 6 months of gestation if not pregnant; CIx: $100–200\times10^6$, AL from women with RSA, i.d.
*[16]	7/11 (64)	2/7 (29)	SIx: 40×10^6 PL from blood of male partner, s.c.; CIx: saline, s.c.
	6/8 (75)		$>150\times10^6$ PL from 1 U blood of male partner, 2/3 i.v., 1/3 i.d., s.c.; CIx: nil
[101]	97/168 (57)		SIx: $58–305\times10^6$–$308–567\times10^6$ PL from 1 U blood of male partner, i.v.
	71/168 (41)		SIx: $568–2{,}667\times10^6$ PL from approx. 10 U blood of male partner, i.v.
[4]	88/106 (83)		SIx: $100–200\times10^6$ X irradiated (50 Gy) PL from 120 ml blood of male partner, i.d. (injected 20 sites on the lateral aspect of the patient's upper arms twice at around 5th and 7th weeks of gestation
	21/38 (55)		SIx: 1×10^6 X irradiated (50 Gy) PL from 1–2 ml blood of male partner, i.d. (injected 20 sites on the lateral aspect of the patient's upper arms at 4 or 5 weeks of gestation. Identical immunization was performed 2 weeks later
[111]	22/29 (66)		SIx: 100×10^6 PL from 100 ml of blood male partner, i.d.; CIx: nil
*[34]	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.
[70]	55/69 (79)		SIx: 40×10^6 PL from 30 ml blood of male partner, s.c., (6–8 sites). Identical immunization was repeated 4 times at intervals of 4–6 weeks before pregnancy; CIx: nil
*[26]	91/136 (66)	60/119 (50)	SIx: $50–60\times10^6$ PL from male partner, s.c.; CIx: saline
[54]	39/48 (81)		SIx: 100×10^6 PL from 100 ml blood of male partner, i.v., i.d., s.c., boost at <6 weeks of gestation; CIx: nil
*[47]	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
[1]	16/21 (76)	13/45 (28)	SIx: 5×10^6 PL from 10 ml blood of male partner, i.d., repeated 6–8 times at 4 weeks interval until APCA became apparent in the cross match; CIx: nil
[104]	19/30 (63)		SIx: PL from 1 U blood of male partner, i.v.; CIx: nil
[57]	4/7 (57)		SIx: $60–80\times10^6$ PL from 80 ml blood of male partner, 1/3 i.d., 2/3 s.c., identical immunization was repeated two times at an interval of 4 weeks; CIx: nil
[113]	47/63 (74)		SIx: $8–10\times10^6$ γ irradiated (20 Gy) PL from 100 ml blood of male partner, s.c.; CIx: nil
[68]	102/117 (87)		SIx: PL from 100 ml blood of male partner, s.c.; CIx: nil
*[51]	154/205 (75)	17/23 (73)	SIx: $100–200\times10^6$ X irradiated (50 Gy) PL from 120 ml blood of male partner, i.d. (injected 20 sites on the lateral aspect of the patient's upper arms at 4 or 5 weeks of gestation. Identical immunization was performed 2 weeks later; CIx: killed streptococcal preparation (KSP), i.d. immunization procedure was same as above
[13]	63/99 (63)	8/50 (36)	SIx: $80–100\times10^6$ 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 weeks intervals until APCA became apparent in the cross match; CIx: NT
	*5/11 (45)	11/31 (35)	SIx: $80–100\times10^6$ 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 weeks intervals until APCA became apparent in the cross match; CIx: NT
[15]	10/23 (43)		SIx: PL from 400 ml blood of male partner, 2/3 i.v., 1/6 i.m., 1/6 s.c.; CIx: nil
[32]	7/9 (77)		SIx: PL from 1 U blood of male partner, 1/2 s.c., 1/2 s.c.; CIx: nil

Table 1 (continued)

Reference of study	Study group	Control group	Immunization protocol
[112]	39/55 (71)		SIx: 100×10^6, PL from 120 ml blood of male partner, s.c., 2 immunization was done at an interval of 8 weeks before pregnancy, boosters were given at 6 weeks intervals during the first 2 weeks of gestation; CIx: nil
*[28]	41/56 (74)	43/61 (71)	SIx: $100–150\times10^6$ PL from 200 ml blood of male partner, i.v., only before pregnancy; CIx: immunization with IgG
[66]	8/10 (80)		SIx: PL from 30 ml blood of male partner, s.c., 4–8 times at an interval of 2 weeks, 1 or 2 booster at an interval of 2 weeks at about 5th week of pregnancy until skin reaction became regressed; CIx: nil
[86]	26/33 (80)		SIx: $50–100\times10^6$ PL from 60 ml blood of male partner, s.c., 2 immunization was done at an interval of 8 weeks before pregnancy, boosters were given at 6 weeks intervals during the first 2 weeks of gestation; CIx: nil
*63	17/20 (86)	17/22 (86)	SIx: PL from 10 ml blood of male partner, i.v., 3 times of more at an interval of 4 weeks; CIx: TPL, from 10 ml blood of other than male partner, i.v., 3 times or more at an interval of 4 weeks
[48]	5/10 (50)		SIx: 50×10^6, PL, i.m. 3 times, at 2–3 weeks intervals, identical booster was given at 2 weeks interval at the end of first trimester; CIx: nil
[90]	5/10 (50)		SIx: PL from 120 ml blood of male partner, 4 equal i.d. injections, 2 injections on each arm. 3 identical immunization was performed at an interval of every 30 days; CIx: nil
[79]	265/413 (64)	81/201 (40)	$80–85\times10^6$ PL from 100 ml blood of male partner, 3/4 i.d., 1/4 s.c., identical immunization was repeated after 3–4 weeks. Booster dose was given when pregnancy was diagnosed; CIx: nil
*[77]	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
[93]	4/92 (58)	7/37 (46)	SIx: PL from 100 ml blood of male partner, i.d. Immunization was performed until the appearance of MLR-Bf in the sera of women with RSA; CIx: NT
[2]	23/28 (82)		SIx: 10×10^6 PL from 10 ml blood of male partner, i.v., i.d., s.c. Immunization was performed 6–8 times at an interval of 4 weeks until the appearance of MLR-Bf in the sera of women with RSA; CIx: nil
[39]	7/12 (58)		SIx: $20–30\times10^6$, PL from male partner, i.d., 3 times at an interval of 4 weeks; CIx: nil
[72]	86/122 (70)		SIx: 245×10^6 PL from male partner, i.d. (injected 8 sites on the forearm of the patients. Patients received 4 courses of therapy at 3 weeks intervals. Booster immunization was performed during first trimester of pregnancy; CIx: nil
[73]	7/10 (70)	47/97 (48)	SIx: $20–30\times10^6$ PL, i.c. 4–6 times at an interval of every 2 weeks until the skin reaction of the injection point had been markedly diminished; CIx: nil
[52]	169/232 (73)		SIx: $100–200\times10^6$ X irradiated (50 Gy) PL from 120 ml blood of male partner, i.d. (injected 20 sites on the lateral aspect of the patient's upper arms at 4 or 5 weeks of gestation. Identical immunization was performed 2 weeks later; CIx: NT
[82]	26/30 (86)	14/43 (32)	SIx: 5×10^6 PL from 10 ml blood of male partner, i.d. (injected at forearm of the women), at 4 weekly intervals maximum up to 6 times before pregnancy; CIx: NT
	*12/14 (85)	2/18 (11)	SIx: 5×10^6 PL from 10 ml blood of male partner was injected (i.d.) 6–8 times at 4 weekly intervals before pregnancy; CIx: 5×10^6 AL, TPL or normal saline was injected (i.d.) 6–8 times at 4 weekly intervals before pregnancy
[81]	67/135(50)		SIx: 5×10^6 PL from 10 ml blood of male partner, i.d. (6–8 times at weekly intervals of 4 weeks, before pregnancy. Identical immunization was repeated at two time points, before pregnancy as well as at each gestation throughout pregnancy; CIx: nil
Total	2,478/3,701 (67)	440/1,198 (36)	

* Randomized trial for lymphocyte immunotherapy in women with RSA ($p<0.01$), pooled data of randomized and nonrandomized trial for lymphocyte immunotherapy in women with RSA ($p<0.001$)

open trial was 86% and 85% in the double blind randomized trial [82]. We also evaluated the efficacy of lymphocyte immunotherapy in two conditions, (once before and once both before and during pregnancy of women with RSA) and found that it was more effective when given twice once before and once during pregnancy [81].

Komlos et al. [57] reported that 7 women with RSA who were vaccinated with the paternal lymphocytes produced significant strong blocking effect of maternal serum on mixed maternal paternal lymphocyte cultures after second vaccination. The extent of the blocking effect in maternal serum and the stimulation in control serum was much higher after immunotherapy in two cases of abortions as compared to cases with normal pregnancy outcome. In addition we have reported the effect of immunomodulation on humoral immune response in women with RSA by comparing the levels of MLR-Bf before and after paternal lymphocyte immunotherapy. It is interesting to note that all 28 women with RSA registered for immunotherapy had an activity index (AI) >1.4 before immunotherapy. Following immunotherapy 23 out of 28 women with RSA developed MLR-Bf and suppressive activity consequently increased bringing

down the AI to <0.5 in all 23 women with RSA. This difference in the pre-immunotherapy and post immunotherapy groups was found to be statistically significant ($p<0.05$). In this study, all 28 women who were registered for immunotherapy conceived within 1 to 6 months after immunotherapy. Twenty-three women (82.15%) continued the pregnancy and have delivered normal infants the remaining 5 women with RSA (17.85%) aborted again in the first trimester [2]. Immunotherapy might react to the decidual T cell recognition of trophoblast and induce a Th2 shift leading to maintenance of pregnancy. Two independent analyses of 15 clinical centers of world wide data of nine randomized trials on allogenic lymphocyte immunization for treatment of women with RSA suggested that alloimmunization may be an effective treatment [94]. However, the data from controlled trials have produced conflicting results regarding treatment effectiveness. Daya and Gunby [26] performed a subgroup analysis of data from a world wide collaborative meta-analysis using the raw data for women with primary RSA entered into randomized controlled trials of immunotherapy which suggests that allogenic immunization is an effective treatment for unexplained primary RSA when pretreatment APCA are absent. Gafter et al. [32] reported that 7 of 9 alloimmunized women with RSA became pregnant and all of them gave birth to live newborns. They reported a decrease in the secretion of the Th-1 cytokine IL-2 and IFN-γ by patient mononuclear cells while NK and LAK cells were markedly decreased. Monocyte function of IL-1, TNF-α, IL-6 and cytotoxic activity decreased concurrently with elevation in IL-10 and TGF-β2 secretion but the production of IL-12 decreased. Recently Hayakawa et al. [39] performed paternal lymphocyte immunotherapy in 12 women with RSA where he demonstrated that this therapeutic approach plays significant role in the maintenance of pregnancy by the shift from Th1 dominant to Th2 dominant status. However, Kwak et al. [61] demonstrated that immunization with paternal lymphocytes suppresses the human NK cell cytotoxicity and CD56+NK cells levels and increases the peripheral blood CD3+ T cell population in women with RSA. These results suggest that improved pregnancy success rates following immunizations may be partly related to the suppression of cell-mediated immunity, monocyte activity and NK cell activity. These data also highlight the possibility that immunotherapy with paternal lymphocytes may give better results when performed before and during pregnancy. Some investigators reported that the success rate of immunotherapy is decreased when autoimmune antibodies (antinuclear antibody, antiphospholipid antibody) and alloimmune antibodies (APCA, Ab2, MLR-Bf) are found to be already present in preimmunized women with RSA [17, 93].

Statistical analysis

In the present study, all outcomes other than live births (continuing infertility, ectopic pregnancy, therapeutic abortion, etc.) were considered to be pregnancy failures. Comparisons of groups were made with analysis of variance and two tailed paired *t*-test. Effectiveness was evaluated in the pooled data by means of the Mantel-Haenzel test. *P* value was considered to be statistically significant if $p<0.02$ or highly significant if $p<0.05$.

Immunization dose used in various clinical trials

We compared different doses and different routes of immunization in various clinical trial of paternal lymphocyte immunotherapy for women with RSA (Table 1). We found that the protection effect of immunization is dose dependent, requiring for a single immunization at least 100×10^6 or $>100\times10^6$ cells for optimal effects. Further, there are some suggestions that the dose administered through intradermal (i.d.) and intravenous (i.v.) routes are most effective. However, lower levels of protection being produced by the subcutaneous (s.c.), intracutaneous (i.c.) and intra muscular (i.m.) routes. Illeni et al. [47] administered 200×10^6 cells and only one-third of the dose was given i.v. studies reporting a favorable effect of immunotherapy used high doses but a lower dose was also used in the studies showing favorable results with immunotherapy [16]. The number of lymphocytes used in immunotherapy might be critical. Less than $60–150\times10^6$ total lymphocytes may result in a suboptimal effect, where as excessive lymphocytes ($>500\times10^6$) may not be effective [16, 101]. Matsubayashi et al. [72] used 245×10^6 and 186×10^6 lymphocytes irrespectively in miscarriage and the successful group. Recent negative results of Ober et al. [77], 200×10^6 lymphocytes were injected at one time and patients not pregnant were immunized at 6 months interval. In a recent positive report of Orgad et al. [79] have reported that $80X10^6$ lymphocytes were injected twice (160×10^6) these findings suggested that the efficacy of immunotherapy may be dependent on how many times performed and how many lymphocytes injected into the women with RSA.

Risks of immunization

The risk of immunization includes transmission of infectious organisms such as the cytomegalovirus, hepatitis B and C viruses and the human immunodeficiency viruses. However, these can also be spread by blood transfusion or use of blood products. The lymphocyte cell suspension should be adequately screened for these infections. It is important to explain the risks involved to the patient while obtaining consent for the procedure, however, adverse side effects caused by lymphocyte immunization, which occur in approximately 1 in 50

treated women [94], are concerned as some are potentially life threatening to the mother and her baby, e.g., hypotension, headache and nausea, etc.

Perlman et al. [85] reported a case of neonatal alloimmune thrombocytopenia after maternal immunization with paternal mononuclear cells. The recurrent miscarriage immunotherapy trialists group reported two cases of neonatal thrombocytopenia out of 1,149 infants whose mothers were treated by allogenic lymphocytes immunization for their unexplained recurrent abortion. However, a recent study present a very rare case of transient neonatal thrombocytopenia found in infant who was delivered by an aborter immunized with paternal lymphocytes once before pregnancy and twice at the 5th and 6th week of gestation for her successful pregnancy. Serological examination revealed that the thrombocytopenia was caused by maternal anti HLA antibodies (anti HLA IgG), which are easily absorbed by various fetal tissues, and its production was induced or enhanced by the paternal lymphocytes immunization [114].

The white cell suspension used for immunization often contains erythrocytes, which may immunize women against paternal blood groups. However, this may happen during normal pregnancy even without immunization. In early pregnancy approximately 50% of women experience vaginal bleeding despite the presence of a fetal heart beat and immunized women have a 7% incidence of intra uterine growth retardation (IUGR) compared to 14% in control patients. There is only 10% incidence of IUGR upon immunizations compared to 30% in control subjects [31]. Therefore, there is evidence that immunization may prevent rather than cause growth retardation. Preterm labor may occur in immunized women.

Conclusion

Alloimmune reproductive failures in women with RSA are psychologically and economically stressful to the childbearing population. The etiology of alloimmune causes of RSA in the vast majority of the cases is not known. However, many recent attempts have been made to understand the role and mechanism of paternal lymphocyte immunotherapy for the treatment of women with RSA. Paternal lymphocyte immunotherapy increased the expression of blocking antibodies (APCA, anti-autologous TCR antibodies, Ab2, MLR-Bf, PIBF) and suppressed Th-1 cytokines and NK cell activity. Research efforts will focus on clarifying the role of blocking antibodies, NK cell, Th1–Th2 cytokines level and their combined effect at the feto-maternal interface.

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