Immune parameters affecting maternal tolerance towards the fetus in normal and aberrant pregnancies

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2

what is wrong with regulatory t cells and foetomaternal tolerance in women with recurrent miscarriages

Abstract

Couples of whom the woman has had a miscarriage have two major concerns: the cause and possible risk of recurrence. Unfortunately, a significant proportion of cases of recurrent miscarriage (RM) remain unexplained despite detailed investigation. Since data suggest that regulatory T cells (Treg) are involved in the maternal acceptance of the allogeneic foetus, RM could possibly be explained by a disturbance of the Treg network. The possible role of Tregs in RM is described in this review, as well as their potential application in diagnostics and therapeutic intervention trials.

Introduction

Approximately 15% of pregnant women experience spontaneous loss of a clinically recognized pregnancy. About 1-2% of couples trying to conceive are confronted with recurrent miscarriage (RM), which is defined as three or more consecutive miscarriages before the 20th week of gestation [1-8]. Several factors influence the risk of miscarriage such as maternal age and previous pregnancy loss. The major known causes include antiphospholipid syndrome, abnormal parental karyotype, endocrine disorders and uterine anomalies [1-7, 9, 10]. However, the cause of RM can only be determined in half of the patients. This burden of continuous uncertainty has a major impact on the lives of women and their partners.

Increasing evidence suggests that the maternal immune response towards the foetus plays a determinative role in the success of pregnancy [9, 11, 12]. Several mechanisms are involved in the induction of maternal tolerance and immunologic acceptance of the semi-allogeneic foetus during pregnancy. Besides the immunological changes occurring locally at the foetal-maternal interface, peripheral immune responses are also altered during pregnancy [9, 13]. Mechanisms for the evasion of the maternal immune response by the foetus include the absence of the classical major histocompatibility complex (MHC) class I antigens human leukocyte antigen (HLA)-A and HLA-B and MHC class II on foetal trophoblast cells preventing allorecognition by T cells and the presence of HLA-C, HLA-E, HLA-F and HLA-G [14-18], preventing allorecognition by natural killer (NK) cells. Furthermore, HLA-G facilitates semi-allogeneic pregnancy by inhibiting maternal immune responses to foreign (paternal) antigens [19]. Another mechanism contributing to immune protection of the foetus is complement inhibition by regulatory proteins decay-accelerating factor (DAF, CD55), membrane cofactor protein (MCP, CD46) and MAC-inhibitory protein (MAC-IP, CD59), and soluble regulators. In addition, trophoblast tissue synthesizes indoleamine 2,3-dioxygenase (IDO), a tryptophan catabolizing enzyme that prevents maternal T cell activation, while galectin-1

(Gal-1) is also expressed at implantation sites, which promotes the generation of tolerogenic dendritic cells [20]. Also the PD1/PDL1 coinhibitory pathway plays a role in foetomaternal tolerance, by limiting the expansion of alloreactive T cells [21]. Other tolerance-inducing cell types highly prevalent in the decidua are CD163+ M2 type macrophages and CD56brightCD16- dNK cells [22]. Another important player in this field is the regulatory T cell (Treg). This heterogeneous subset of T cells suppresses the induction and proliferation of effector T cells and plays an essential role in the sustainability of peripheral immune tolerance [23-26]. The mother's acceptance of the foetus, which can be seen as an allograft expressing paternally inherited alloantigens, during pregnancy is a unique example of how the immune system reshapes a destructive alloimmune response to a state of tolerance. Therefore, knowledge on the role of Tregs in successful and aberrant pregnancy may also be relevant for cell and organ transplantation as acceptance of the allograft is a desirable goal in both reproductive immunology and transplantation.

In this review, the role of Tregs in foetal-maternal immune tolerance as well as in recurrent miscarriage will be discussed. The subject of preeclampsia will not be addressed in this review, because of the difference in pathophysiology. Understanding the complex mechanisms of foetomaternal tolerance has important implications for developing novel strategies to induce immunologic tolerance in humans in general and for prevention of spontaneous abortion in high-risk populations in particular.

Regulatory T cells: Phenotype and function

Regulatory T cells play a pivotal role in controlling adaptive immune responses and maintaining self-tolerance. This unique subpopulation of T cells has shown to be involved in preventing autoimmunity, and tolerating allogeneic organ grafts in rodent models. The suppressive activity of Tregs is mediated either in a cell-cell contact mediated fashion via cytotoxic T lymphocyte-associated protein 4 (CTLA-4) or by the secretion of cytokines such as transforming growth factor beta (TGF-β) or interleukin (IL)-10 [27-31]. In 1995, Sakaguchi characterized a subpopulation of T cells with suppressive capacity [32]. These regulatory T cells were then described as being CD4+CD25+ T cells, a phenotype definitely not unique to Tregs. Ever since, a major obstacle to the study and application of Tregs in the human setting has been the lack of specific cell surface markers to define Tregs and separate them from other T cell subsets [33, 34]. The transcription factor forkhead box P3 (FoxP3) was considered as a specific marker for Tregs essential for their thymic development, phenotype, and function [35-37]. Although Foxp3 is expressed exclusively by Tregs in mice, Foxp3 expression in humans occurs in immunosuppressive Tregs as well as in recently activated effector T cells, and thus does not specifically identify human regulatory T cells [38-40]. To address this limitation, high expression of CD25 and downregulation of the IL-7 receptor (CD127), along with intracellular Foxp3 expression, have been used as phenotypic markers for regulatory T cells. Several investigators confirmed that isolation of T cells with high expression of CD25 and low expression of CD127 will result in a highly purified population of Tregs with suppressive capacities in functional assays [33, 35, 41-43]. Other markers that have been associated with (certain subsets of) Tregs are Helios, CTLA-4, CD45RA/RO, CD62L, C-C chemokine receptor type 6 (CCR6) and CD39 [28, 43, 44]. Tregs are comprised of two main populations: thymus-derived natural Tregs and peripherally generated induced Tregs [45]. However, in most studies concerning Tregs in recurrent miscarriages no distinction was made between both populations.

Tregs in normal pregnancy

Tregs in rodent models of pregnancy

In 2004, Aluvihare and colleagues were the first to report that Tregs are required for the maternal immune system to tolerate a foetal allograft in mice [46]. They showed an unusually high proportion of CD4+CD25+ Tregs in almost all tissues of pregnant mice compared to non-pregnant mice, independent of the presence of a paternal MHC difference. Treg frequencies in blood of mice increased during early pregnancy, progressively decreased from mid-gestation onwards, and at term returned to levels that are comparable to non-pregnant conditions [47]. This indicates that the maternal immune system undergoes a systemic change during pregnancy. In addition to the expansion of Tregs in pregnant compared to non-pregnant mice, a diminished number and function of Tregs was found in abortion-prone animals [48-50]. These animals expressed even lower levels of CD4+CD25+ Tregs than age-matched non-pregnant control mice. The abovementioned results suggest a crucial role for Tregs in avoiding immunological rejection of the foetus.

To test whether Tregs are indispensable for maternal immune tolerance toward the foetus, adoptive transfer experiments in mice were performed [46, 51]. Transfer of lymphocytes depleted of CD4+CD25+ Tregs into pregnant T-cell-deficient mice led to gestation failure. Additionally, the adoptive transfer of pregnancy-induced Tregs into abortion-prone mice prior to mating significantly increased IL-10 and TGF-β mRNA expression in decidua and lowered the foetal resorption rates [48, 51]. This suggests an active and essential role for Tregs in mediating maternal tolerance to the foetus. Importantly, this treatment was only successful if applied at an early stage of pregnancy, and transfer of Tregs from non-pregnant mice to the abortion-prone mice was ineffective [50, 52]. Blocking regulatory T cell function by an anti-CD25 monoclonal antibody (mAb) on day 0 of pregnancy in normal pregnant mice inhibited implantation, while anti-CD25 mAb treatment later in pregnancy reduced Treg cell numbers, but did not induce any parameters reflecting abnormal pregnancy [48, 50, 53]. These findings suggest that Tregs are important to mediate maternal tolerance to the allogeneic foetus in the implantation phase and early stages of pregnancy, while Tregs may not be required for maintenance of the late stage of allogeneic pregnancy.

Although Aluvihare and colleagues argued that expansion of Tregs during pregnancy is alloantigen-independent [46], Zhao and colleagues reported that frequencies of CD4+CD25+ Tregs increase to greater extent in allogeneic than in syngeneic pregnancies in mice [47]. In addition, Shima *et al*. showed that administration of anti-CD25 mAb early in pregnancy induced implantation failure in allogeneic pregnant mice, but not in syngeneic pregnant mice [53]. These results suggest an involvement of paternal antigens in Treg expansion.

Treg induction by seminal plasma

The stages wherein Tregs specific for paternal antigens develop are not yet fully defined. There is evidence that seminal plasma may already induce a tolerogenic environment.In mice, paternal antigens and maternal MHC class II cells can be found in the vaginal mucus already within the first hours of pregnancy [54], indicating the possibility of local antigen presentation at very early stages. Exposure of the mouse female genital mucosa to seminal plasma induced the expansion of CD4+CD25+FoxP3+ Tregs in the lymph nodes draining the uterus, promoting tolerance to paternal alloantigens [55-57]. The increase in CD4+CD25+ cells was abrogated when seminal vesicles were excised before mating [55, 56]. Immediately after insemination, paternal antigens were found in several organs of the female mice [54, 57]. This emphasizes the possibility that Tregs proliferate after encountering semen-derived paternal antigens presented on antigen-presenting cells (APCs) in secondary lymphoid organs. More specifically, a soluble form of

CD38 (sCD38) released from seminal vesicles to the seminal plasma might play a role in this process. Soluble CD38 in seminal plasma was shown to be crucial for the induction of uterine tolerogenic dendritic cells (DCs) and CD4+Foxp3+ Tregs [58]. Deficiency of sCD38 in seminal fluid increased the loss rate of allogeneic foetuses, which could be rescued by a direct injection of recombinant sCD38 into the uterus. The immunoregulatory role of seminal plasma is not exclusive to rodents. Exposure of human peripheral blood T cells to seminal plasma *in vitro* led to increased mRNA expression of CD25, IL-10 and FoxP3, which was partly dependent on the presence of APCs [59]. These results suggest that seminal plasma contains immunomodulatory factors that may contribute to the formation of a tolerogenic environment at the embryo implantation site and that exposure to seminal fluid at mating promotes a state of functional tolerance mediated by expansion of the local antigen-specific Treg pool. One of these immune modulating aspects in semen could be soluble HLA (sHLA), as human seminal plasma contains sHLA-G and sHLA class I [59, 60]. HLA-G inhibits the proliferation and cytotoxic functions of T cells and induces immunosuppressive T cells [15-17, 61]. Peptides derived from the paternal HLA class I antigens in the seminal plasma may be presented by maternal APCs in the endometrium and when the proper cytokines are present in the seminal fluid this may lead to the induction of regulatory T cells (Figure 1). Several prostaglandins, cytokines and chemokines have been described to be present in seminal plasma, such as pro-inflammatory IL-1, IL-6, IL-8, tumor necrosis factor (TNF)-α, interferon (IFN)-γ, granulocyte macrophage colony-stimulating factor (GM-CSF) and chemokine C-X-C motif ligand (CXCL)1, and anti-inflammatory prostaglandin E2 (PGE2), TGF-β, CXCL10, chemokine C-C motif ligand 17 (CCL17), MCP-1 and macrophage colony-stimulating factor (M-CSF) [62-66]. Increasing evidence suggests that proteins in seminal fluid are able to interact with the vaginal, cervical and uterine epithelium to elicit a series of changes in the immune responsiveness of the female [67-69]. However, seminal plasma shows great variety between men in the concentrations of cytokines and in the strength and quality of the cytokine response elicited [68]. This diversity might influence the maternal immune response. A profile with high levels of regulatory proteins, such as TGF-β and PGE2, can contribute to the secretion of inhibitory cytokines TGF-β, IL-10 and IL-35 by maternal APCs. The secretion of these cytokines can lead to the suppression of activation and expansion of conventional T lymphocytes and the induction of maternal regulatory T cells and tolerogenic DCs (Figure 1; cytokine profile A), whereas a cytokine profile with high levels of pro-inflammatory cytokines, such as GM-CSF and IL-8, might induce a response eliciting the expression of pro-inflammatory cytokines and chemokines and the recruitment of macrophages, dendritic cells, and lymphocytes (Figure 1; cytokine profile B). This inflammatory response might lead to pregnancy complications or even pregnancy loss.

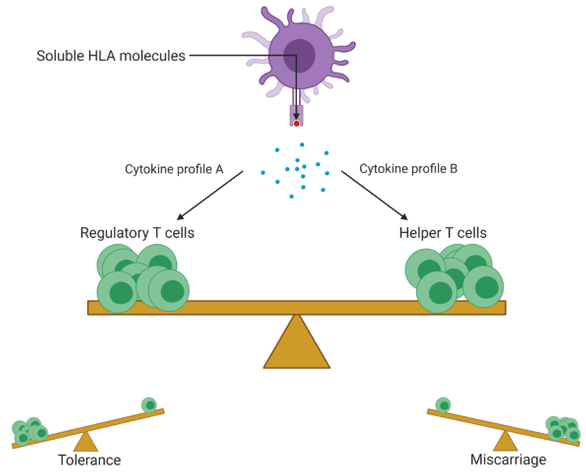

Figure 1. Soluble HLA molecules and cytokines may affect the local immune response during implantation. Paternal HLA antigens, present in seminal plasma in the form of sHLA, might be taken up and presented by maternal APCs. These APCs present the allogeneic peptides to naïve T cells. The cytokine environment present at the time the paternal antigens are first encountered is pivotal in controlling differentiation of APCs, which can determine the strength and quality of the ensuing T cell response. Many cytokines are present in seminal plasma. The specific cytokine profile in seminal plasma varies between semen samples. When regulatory proteins, e.g. TGF-β and PGE2, are present in the seminal fluid, this can contribute to the secretion of inhibitory cytokines TGF-β, IL-10 and IL-35, which can lead to the induction of specific regulatory T cells and a tolerogenic environment (cytokine profile A). On the other hand, the cytokine profile in the seminal plasma can contribute to the promotion of a Th1 like response, which can lead to activation and expansion of conventional T lymphocytes and pregnancy complications (cytokine profile B). Adapted due to poor quality of original figure. Created with BioRender.com.

Tregs in human pregnancy

In humans, dynamic changes in circulating CD4+CD25+ Treg frequencies during pregnancy have been found, similar to what was seen in mice: a marked increase during early pregnancy, peaking during the second trimester, and a progressive decrease to levels comparable to non-pregnant conditions at term [47, 70-72]. Svensson-Arvelund *et al*. showed that human foetally derived placental tissue promotes the induction of suppressive CD25highCD127lowFoxp3+ Tregs within tissue *in vitro*, in parallel with increased IL-10 production [22]. The expansion of Tregs was mediated, in part, by TGF-β and IL-10, produced particularly by trophoblast cells. Galectin-1, a progesterone regulated protein expressed at the foetomaternal interface, also induces the expansion of CD4+CD25+FoxP3+ Tregs[73]. In addition, the PD1/PDL1 pathway promotes both the induction and maintenance of CD4+Foxp3+ regulatory T cells, where PDL1 is expressed by foetal cells and PD1 is expressed by maternal cells [74-76]. A novel inhibitory cytokine identified to play a role in the regulation of maternal-foetal immune tolerance is IL-35. This cytokine is produced primarily by CD4+Foxp3+ Tregs and is required for maximal suppressive activity of Tregs *in vitro* and *in vivo* [77, 78]. It has been reported that first-trimester human trophoblast cells express and secrete IL-35, which might contribute to their suppressive capacity toward maternal immune cells [79]. Interestingly, it was found that the level of IL-35 was significantly higher in pregnant females compared to age-matched non-pregnant females, which may suggest that increased IL-35 in pregnancy provides immune protection for the foetus [77]. In this way, by several placental factors acting in concert, the foetal placenta is able to create a tolerant uterine environment.

The induction of labour in humans is associated with a decrease of peripheral CD4+CD25high Tregs and a sharp increase of peripheral CD4+CD25low T cells [47], the latter largely representing activated effector T cells. Tilburgs *et al*. also observed this significant increase in the CD4+CD25low T cell fraction in maternal peripheral blood lymphocytes at term pregnancy compared to peripheral blood of early pregnancy subjects and to peripheral blood of non-pregnant controls. However, they did not observe significant differences in the level of peripheral CD4+CD25high T cells in early pregnancy, term pregnancy, and non-pregnant controls [80]. When comparing the decidua to maternal peripheral blood and peripheral blood of non-pregnant controls, a significantly higher percentage of CD4+CD25high T cells was found [13, 80]. Furthermore, these CD4+CD25high T cells from the decidua contained a significantly higher suppressive capacity to regulate the maternal immune response to foetus-specific UCB cells compared to CD4+CD25high T cells in maternal blood [13]. These results suggest that foetus-specific Tregs are specifically recruited from the periphery to the foetal-maternal interface. Sindram-Trujillo *et al*. compared the immune cell composition of decidua collected after spontaneous vaginal delivery to elective caesarean section without labour. Labour appeared to be associated with dynamic changes in the distribution of decidual leukocytes, specifically NK and T cell subpopulations. The percentage of CD3+CD4+CD25+ cells in the decidua basalis and decidua parietalis after spontaneous vaginal delivery was lower than after caesarean section [81]. This down-regulation of Tregs might lead to an abnormal immune milieu, which confers susceptibility to pregnancy loss. Hence, low Treg levels may be associated with recurrent miscarriages.

Tregs in recurrent miscarriages

Before pregnancy

Compared to non-pregnant women, peripheral CD4+CD25high Tregs are increased in healthy women early during pregnancy. However, they are decreased in women with (recurrent) miscarriages compared to normal early pregnancy, at a level comparable with that of non-pregnant controls [70, 82, 83]. This difference in

Treg level can already be detected in non-pregnant women with RM. It has been shown that Treg frequencies undergo profound changes during the menstrual cycle [84]. Fertile women showed an expansion of Tregs in the late follicular phase followed by a dramatic decrease in Treg frequencies in the luteal phase of the menstrual cycle, whereas women with RM had similar Treg frequencies at both phases. At both the follicular and luteal phases, decreased frequencies of peripheral Tregs were observed in women with unexplained RM compared to fertile controls [85, 86]. These low levels of Tregs were similar to peripheral Treg numbers in postmenopausal women [86]. This may suggest that reproductive failure results from the inability of Tregs to sufficiently expand during the pre-implantation phase. Furthermore, infertile women have significantly reduced Foxp3 mRNA levels in the endometrium, supporting the concept that unsuccessful pregnancy is caused by the lack of sufficient Tregs [87]. In healthy women, Tregs are capable of regulating effector T cells that respond to paternal antigens. A lack of regulation thus may also be detected by high levels of paternal antigen-specific effector T cells. Indeed, when women with RM were compared to controls, the frequency of sperm antigen specific effector T cells was higher and accompanied by a lower frequency of sperm antigen specific Tregs [88]. Furthermore, these sperm specific Tregs in women with RM expressed less Ubc13, which is a critical molecule preventing Tregs from differentiating into effector T cells [88, 89]. Knockdown of Ubc13 from isolated Tregs converted the Tregs to effector T cells.

During pregnancy

Lower proportions of CD4+CD25high T cells with FoxP3 expression are found in peripheral blood and decidua from pregnant women with RM compared to those with normal early pregnancies [85, 90-93]. This suggests that women with unexplained RM are less capable to induce and maintain immune tolerance towards foetal alloantigens. Furthermore, it has been shown that the level of IL-17+ T cells and ratio of IL-17+ T cells/Tregs was significantly increased in peripheral blood from non-pregnant women with unexplained RM when compared with fertile controls [85, 93]. Th17 cells can exert a rapid response at sites of inflammation and may play a role in allograft rejection in solid organ transplantation [94, 95]. Likewise, trophoblast invasion from the allogeneic foetus and the shedding of foetal antigens may stimulate a maternal systemic inflammatory response and may therefore cause the emergence of Th17 cells [94]. This suggests that an immunologic imbalance and subsequent immune dysregulation by the altered Th17/Treg cell populations influences pregnancy outcome.

When compared with specimens obtained from abortions on social indication, the proportion of decidual CD4+CD25high T cells in products of conception from miscarriages was significantly lower [96]. This confirms that decidual CD4+CD25high T cells are likely to contribute to the mechanisms mediating maternal immune tolerance and maintenance of pregnancy. In addition to the decreased frequency of CD4+CD25+CD127low Tregs in unexplained RM decidua compared to controls, the suppressive activity of CD4+CD25+CD127low cells on effector T cell proliferation was impaired in unexplained RM decidua [97]. Higher Treg numbers were required to exert a similar magnitude of *in vitro* suppression, mediated predominantly through TGF-β and IL-10, compared to CD4+CD25+FoxP3+ cells from fertile women [86, 97]. The expression of intracellular TGF-β and IL-10 in Tregs was lower in the RM group than in the control group [97]. As mentioned before, IL-35 is required for maximal suppressive activity of Tregs *in vitro* and *in vivo* [66, 67], and whereas this cytokine was increased in normal pregnancy, it was decreased in RM women [77]. Also galectin-1 expression was decreased in women with RM compared to healthy early pregnant women [98].

Women with RM having low CD4+CD25+Foxp3+ Treg levels in the first trimester

experienced a significantly lower ongoing pregnancy rate than those with a higher Treg level in the first trimester [99]. The decreased expansion of Tregs during pregnancy in the unexplained RM group may predispose to pregnancy loss, and Tregs might serve as a pregnancy marker to aid in predicting miscarriage risk in newly pregnant women. [99, 100]. Furthermore, this highlights the opportunity to use Treg therapy to increase the success rate in women who repeatedly experience pregnancy losses.

Tregs as a therapy for recurrent miscarriages

Trials on the use of Tregs to treat graft-versus-host disease (GvHD) in patients with a stem cell transplant showed acceptable safety and promising efficacy, e.g. reduced incidence of severe acute GvHD [101-104]. This has led to the use of Tregs in other fields as well. Whereas studies in solid organ transplantation are already focusing on safety [105], the administration of Tregs has not yet been applied to pregnancy. However, immunotherapeutic procedures that indirectly increase Tregs to prevent maternal rejection of the foetus have been introduced. These immunotherapies include boosting the maternal immune response by paternal (woman's partner) or third-party (donor) lymphocyte immunization. Alternative immunotherapies include products derived from early embryos (trophoblast membranes) or antibodies derived from blood (immunoglobulin therapy). Paternal or third-party lymphocyte immunization has been the most widely used treatment for alloimmune-mediated miscarriages. However, this therapy is still controversial in terms of effectiveness. The latest Cochrane review by Wong *et al*. showed that none of these treatments provided a significant beneficial effect over placebo in improving the live birth rate or reducing the risk of future miscarriage in women who had RM [106]. Nevertheless, some studies showed that the proportion of CD4+CD25high T cells in peripheral blood from women with unexplained RM was significantly increased after paternal or third-party lymphocyte immunization therapy [107-109], and 80-90% of patients who underwent immunotherapy successfully delivered a baby [109]. Furthermore, the proportion of Tregs was significantly higher in successfully pregnant women than in those with pregnancy loss after lymphocyte therapy [107-109]. In those who experienced an unsuccessful pregnancy, no significant change of the proportion of CD4+CD25+ T cell/PBMC and CD4+CD25+CD127-/CD4+ T cell was observed and the level of Tregs remained low. After successful immunotherapy, the percentage of Th17 cells was significantly lower and the Th17/Treg ratio significantly decreased to a level comparable to that before immunotherapy. Unfortunately, in these studies it is not uniformly described whether lymphocyte immunization was performed with cells of the partner or a third-party.

Other therapies that intend to induce Tregs in women with unexplained RM involve the administration of cytokines and hormones. Scarpellini and Sbracia tested the use of granulocyte colony-stimulating factor (G-CSF) in women with unexplained RM [110]. G-CSF is a cytokine that, amongst others, can recruit and activate tolerogenic dendritic cells, which can aid in the generation of Tregs [9, 111, 112]. G-CSF treatment showed an evident effect on the pregnancies of women with RM, with a remarkable increase in success rate and a consequent reduction of miscarriages. Currently, the RESPONSE trial is testing the effect of G-CSF administration in women with three or more unexplained miscarriages in a randomised, double-blind, placebo-controlled trial (NCT02156063). Also, progesterone is suggested to be an important regulator of systemic and local Treg development and function [113, 114]. For now, it is still unclear whether it is effective in women with RM [115, 116]. The report of a large multicentre study (PROMISE) of progesterone supplementation for RM is currently awaiting publication (ISRCTN92644181).

Conclusions

Tregs have a critical role in maintaining immune tolerance to self-antigens and to foreign antigens of the semi-allogeneic foetus: a deficiency in Tregs is associated with implantation rejection at early stages of pregnancy and abortion. Whether immunotherapy can play a role by preventing maternal rejection of the foetus has yet to be established, but modulation of the immune system as (part of) a therapeutic strategy is certainly a valid option to prevent recurrent miscarriages.

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