Adoptive transfer of Tregs: a novel strategy for cell-based immunotherapy in spontaneous abortion: lessons from experimental models

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Abstract

Since half of the genes are inherited from the paternal side, the maternal immune system has to tolerate the presence of foreign paternal antigens. Regulatory T cells facilitate the development and maintenance of peripheral tissue tolerance of the fetus during pregnancy. Reduction in regulatory T cells is associated with complications of pregnancy, including spontaneous abortion. Recent studies in mouse models have shown that the adoptive transfer of Tregs can prevent spontaneous abortion in mouse models through improving maternal tolerance. Thus, adoptive cell therapy using autologous Tregs could potentially be a novel therapeutic approach with cell-based immunotherapy in women with unexplained spontaneous abortion. Besides, strategies for activating and expanding antigen-specific Tregs *ex vivo* and *in vivo* based on pharmacological agents can pave the foundation for an approach incorporating immunotherapy and pharmacotherapy. This review aims to provide an evaluation of the current understanding of the therapeutic potential of the adoptive transfer of Tregs in the treatment of spontaneous abortion disease.

Keywords: Immune tolerance; Regulatory T cell; spontaneous abortion; Adoptive cell therapy

Introduction

The fetus is a semi-allogeneic graft; half of its MHC molecules come from the maternal side and half from the paternal side. Hence, the fetus is antigenic while the mother is immunologically responsive [1]. Successful pregnancy needs immune tolerance from mother to enable implantation of the semi-allogeneic fetus (with paternal antigens) during the gestation [2-4]. If this immunological tolerance breaks down complications of pregnancy such as spontaneous abortion may occur [5, 6]. Spontaneous abortion is defined as a pregnancy loss at < 20 weeks of gestation in the absence of elective surgical or medical measures to terminate the pregnancy [7].

Regulatory T cells (Tregs) are a subset of immune cells that regulate the immune responses [3]. Impaired synthesis or function of Tregs can cause autoimmunity or rejection of allografts [8, 9]. Accumulating evidence suggests that Tregs contribute to an enhanced maternal tolerance towards the fetus antigens [6, 10, 11].

Animal models are useful for understanding the biological mechanisms of various disease processes. The mating of CBA/J females with DBA/2J males results in an abortion prone mouse model known as CBA/J× DBA/2J. The characteristic features of these mice are smaller sized embryos, implantation site hemorrhage and necrosis [12]. CBA/J mice share many features with human abortion and have been a well- known model of recurrent spontaneous [13]. Abortion prone mouse has spontaneously high abortion rates (20-40%) [12]. Use of the mouse model of spontaneous abortion can lead to significant advances in the understanding of immune mechanisms contributing to spontaneous abortions as well as any potential clinical interventions [12]. In this study, we reviewed the existing knowledge of immunotherapy on the

adoptive transfer of fresh or *ex vivo* expanded Tregs in the treatment of spontaneous abortion in related mouse models.

An overview of the T cell immune response network in spontaneous abortion

T-cell mediated adaptive immune responses play a crucial role in pregnancy outcomes. A complex network including natural killer-T (NKT) cells, cytotoxic T cells and cytokines is essential for successful implantation and pregnancy [4, 14, 15].

It has been shown that various subsets of $CD4^+$ T-helper cells control immune responses as a network. $CD4^+$ T cells include T helper 1 (Th1), Th2, Tregs and Th17 cells. These cells play a crucial role in the feto-maternal immunity [16-18]. The survival of a fetus in the uterus of humans and murine relies on the Th1/Th2 cytokine balance [19]. There is a predominance of Th2 cells that secrete IL-4 and IL-10 during normal pregnancy whereas there is a predominance of Th1 cells that produce IFN-γ and TNF-α and in pregnancies associated with recurrent spontaneous abortion [10, 20]. High levels of IFN-γ and TNF-α in the peri-implantation period are associated with adverse effects on the development of the placenta and fetus [6].

In the CBA/J×DBA/2 model, there is reduced production of IL-4 and IL-10 by whole placenta cultures, lower expression of IL-4 and IL-10 expression in the placental tissues [21, 22] and increased Th1-type systemic responsiveness of CBA/J maternal T cells compared with normal pregnancies (CBA/J × BALB/c) [23]. Various studies demonstrate that IFN-γ is increased in abortion prone mice [24-27]. Furthermore, administration of IFN-γ, TNF-α [21] and Th17 cytokine (IL-17 A) [28] resulted in significantly increased resorption rates [21, 28].

However, Th1/Th2 balance by itself is not sufficient to explain the mechanism by which maternal immune cells tolerate fetus. Studies have demonstrated that the proportion of Th17 ($CD4^+IL\text{-}17A^+$) cells in both peripheral blood and decidua was significantly higher in women undergoing spontaneous abortion than that in normal pregnant women [16] as well as in abortion prone mice model [29].

Human Th17 cells producing IL-17 located in cyto- and syncytiotrophoblasts play a major role in rejecting fetal antigens [30]. IL-17, as a pro-inflammatory cytokine, plays a fundamental role in the pathogenesis of preterm labour and miscarriage [31]. In the CBA/J × BALB/c mouse model of normal pregnancy, injection of transvaginal IL-17, could increase the abortion rate, as well as reduce the expression of TGF-β and IL-10 without any effects on the expression of IFN-γ and IL-4. This suggests that overexpression of IL-17 alone induces inflammation which leads to an imbalance in the immune tolerance in the decidua [28].

Tregs suppress proliferation and cytokine secretion from pro-inflammatory Th1 and Th17 cells which typically secretes pro-inflammatory IFN-γ and IL17, respectively [32]. The role of Tregs in the maintenance of maternal tolerance is described in the following sections.

Tregs

It was shown in the first time in 1995 that a subset of thymus-derived $CD4^+$ T cells expresses high levels of CD25 (α chain of IL-2 receptor) which protects thymectomized mice from autoimmunity [33]. Since then, there is growing evidence that Tregs play a crucial role in the maintenance of immune homeostasis [34] and suppression of autoimmunity [35]. Around 5–15% of peripheral CD4 + T cells in humans and mice are composed of Tregs. Tregs play a

pivotal role in the maintenance of self-tolerance or peripheral tolerance [34, 36, 37]. The specific markers of Tregs are $CD4^+$, $CD25^{+high}$, Foxp3, cytotoxic T lymphocyte-associated protein 4 (CTLA4), programmed cell death 1 (PD-1) and $CD127^{low}$ [34]. The CD127 expression on mouse Tregs differs depending on their location and activation phase [38].

Some of the immature $CD4^+$ T cells reorganize as self-antigen (auto-antigen) with high affinity differentiate into Tregs [so-called natural Tregs (nTreg)] during the development of T cells [34, 36]. Recognition of self or exogenous antigens in the absence of inflammation promotes the differentiation of inducible Tregs (iTregs) from naïve $CD4^+$ T cells in the peripheral tissues [34]. There two phenotypically different immunosuppressive subtypes of the iTregs are the IL-10 producing T regulatory type 1 (Tr1) cells and the TGF-β-producing Th3 cells [39, 40].

Tregs acts by cell-cell contact mediated by signalling via the negative regulator of T-cell activation (CTLA- 4) and secretion of the key cytokines such as TGF-β and IL-10 [41-44]. TGF-β is a multifunctional cytokine secreted by several types of immune cells, including Tregs. TGF-β has been shown to maintain the peripheral natural Tregs that develop in the thymus and induces the differentiation of naive $CD4^+$ T cells to Tregs. TGF-β is also important in the differentiation of Th17 cells. Naive CD4+ T cells differentiate into Th17 cells in the presence of TGF-β and IL-6 [45]. TGF-β amplifies the function of Tregs *ex vivo,* suggesting that TGF-β is involved in the physiology of Tregs [25]. TGF-β deficiency or inhibition *in vivo* eliminates the suppressive activities of Tregs [25]. These findings suggest that the immunosuppressive activity of Tregs are dependent on TGF-β.

Tregs inhibit the activation, maturation and function of macrophages and DCs, which are the two important cells of the innate immunity (i.e. macrophages and DCs). This inhibition is through the production of anti-inflammatory cytokines such as IL-10 and TGF-β [40], the disruption of metabolic pathways and also the consumption of IL-2. IL-2 is a key cytokine for the proliferation and differentiation of other T cells. It also suppresses the activation, proliferation, differentiation, and function of T and B cells [34, 36]. Activated Tregs also interact with DCs through CTLA4 resulting in the down-regulation of DC co-stimulatory molecules (CD80 and CD86) which cause effector T cells activation [46].

Tregs and spontaneous abortion

The regulation of immunity is vital for a successful pregnancy [47]. Tregs play a pivotal role in maintaining maternal tolerance [6, 29, 40] of the fetus during pregnancy. In both mice and humans, Tregs will peak in the second trimester of pregnancy and then diminish during the later stages of pregnancy and the postpartum period [48-51]. Parental antigens, co-stimulatory molecules, alterations in the human pregnancy hormones such as human chorionic gonadotropin (HCG) are involved in the expansion of Tregs during pregnancy [52]. There are growing evidence that deregulation of the number and function of Tregs in decidua and peripheral blood could lead to spontaneous abortion in humans and mice [10, 16, 29, 53-58].

Tregs contribute to successful pregnancy via suppressing self-reactive lymphocytes through producing cytokines such as TGF-β and interleukin IL-10 [41, 42, 53, 59]. IL-10 had a pivotal role in the maintenance of maternal-fetal tolerance [60, 61]. It has been shown that the IL-10 null

mutant mice are prone to inflammation-induced fetal loss and the treatment of abortion-prone mice with IL-10 resulted in the prevention of fetal loss [21].

TGF-β is mainly secreted by CD4+ T cell subsets especially Tregs. TGF-β is expressed in endometrium and gestation decidua [62]. TGF-β level changes during pregnancy and spontaneous abortion and have a pivotal role in both promoting and limiting placental development [4, 62, 63]. It can inhibit the proliferation of T cells and the activity of cytotoxic T lymphocytes and natural killer cells, thereby reducing embryotoxicity [62]. Interestingly, Tregs suppress Th1 and Th17 cells by inhibiting the production of cytokines.

The abortion rate was correlated with reduced levels of IL-10^{+} Tregs as well as the lower FOXP3 expression. This was associated with elevated levels of Th1 IFN-γ^{+} cells in the CBA/J×DBA/2 model compared to the decidua of healthy pregnant mice [13]. Additionally, IL-10^{+} Tregs levels in the thymus were lower compared to the controls suggesting that higher levels of $CD4^{+}CD25^{+}$ Treg were produced during normal pregnancy in the thymus in comparison with miscarriage [13].

Uterine mast cells (uMCs) is a subset of innate immune cells in uterus involved in the implantation by remodeling spiral arteries (which is necessary for enhancing maternal blood flow to the fetal side), promoting angiogenesis, placenta size and fetal development [64]. It has been shown that lower Treg numbers correlate with lower uMC numbers in abortion prone mice demonstrating the interplay between Tregs and uMCs in the normal development of pregnancy. Therefore Tregs may affect maternal vascular remodeling and early placental

development [65]. Taken together, Tregs are key mediators for maintaining maternal tolerance. The immunoregulatory mechanism of Tregs at the feto-maternal interface shown in figure 1.

Adoptive transfer of Tregs and spontaneous abortion

Adoptive transfer of Tregs potentially is an effective strategy to treat Treg-mediated diseases. This includes isolating *in vivo* differentiated Tregs or expanding Tregs *ex vivo* or generating iTreg cells *in vitro,* and subsequent transfer into the body (37). Therapeutic interventions for enhancing tolerance based on the adoptive transfer of Tregs in animal models and clinical trials have been already established in autoimmunity and tissue transplantation [66-69]**.**

In the 2005 year, Zenclussen et al. published the first research in the context of adoptive transfer Tregs in DBA/2-mated CBA/J mice as the abortion prone mouse model [13]. Adoptive transfer of fresh Tregs caused a significant reduction in the abortion rate and a significant up-regulation of IL-10 mRNA expression in decidua and placenta of abortion-prone mice [13]. This study suggested that an accumulation of Tregs at the fetal-maternal interface could result in the prevention of abortion [13]. Interestingly, after transfusion of Tregs from normal pregnant and non-pregnant CBA / J mice, proliferation and IFN-γ secretion of Th1 cells from abortion mice *in vitro* decreased, while *in vivo* prevention of abortion could only occur after adoptive transfer of Tregs from normal pregnant mice. Therefore, the key finding of this study was that pregnancy-induced Tregs play a crucial role in maternal tolerance to fetal antigens [13]. While the next study that carried out by Yin et al. showed that adoptive transfer of *in vitro* expanded Tregs of non-pregnant CBA/J mice on day one drastically diminished abortion rates that were associated with increased ratios of serum IL-10/ IFN-γ and TGF-β1/ IFN-γ in the mouse model of spontaneous abortion [26]. This study showed that irrespective of the time of transfusion of

Tregs during pregnancy, adoptive transfer of freshly isolated Tregs had no significant effect on abortion rate in abortion mice compared to the control mice without transferring of Tregs [26]. This finding might be clarified by using more suppressive *ex vivo* expanded Tregs than freshly isolated cells [70 67]. The use of different numbers of Tregs probably could affect the results of the transfusion of Tregs [13, 26].

In a different setting from the mentioned studies [13, 26] Wang et al. showed that transvaginal rIL-17 (10 μg/mouse) into CBA/J × BALB/c mouse (model of normal pregnancy) caused abortion [28]. Adoptive transfer of pregnancy-induced Tregs isolated from decidua of normal pregnant CBA/J mice, stimulated with immobilized anti-mouse CD3 antibody and anti-mouse CD28 antibody in the presence of recombinant mouse IL-2 before mating reduced the abortion rate and increased IL-10 and TGF-β levels in decidua in the mouse model of normal pregnancy [28]. However, the transfer of Tregs did not affect IFN-γ or IL-4 expression in the decidual tissue. The authors concluded that Treg therapy has potential applications in the prevention of abortion induced by inflammation in the normal pregnancy model before mating happens [13, 28].

It has been shown that $CD117^{+}Fc\varepsilon\ RI\alpha^{+bright}$ uterine mast cells involved in the implantation through the remodeling of spiral arteries and improving angiogenesis via reduction of soluble fms-like tyrosine kinase 1 (sFlt-1) level (an anti-angiogenesis factor) leading to enhance placentation, placenta size as well as fetal growth at the fetal-maternal interface [71]. The frequency of uterine mast cells increases and remains high during early gestation when the frequency of Tregs increases. It has been documented that low frequency of Tregs correlates with the low frequency of uMC numbers in abortion mice [65].

Woidacki et al. showed that the adoptive transfer of freshly isolated Tregs into CBA/J × DBA/2J combination during early pregnancy (day 0 of pregnancy, after plug detection) was associated with a diminished abortion rate and a rise in the proportion of uMCs in the placenta, in the oviduct and the splenic tissue as well as a decrease in sFlt-1 levels. These alternations helped to improve the remodeling spiral artery and increased placenta size [65]. Interestingly, IL-3 and mSCF (growth factors for mast cells) [71] increased after the transfer of Tregs suggesting that Tregs act by augmenting these two mediators, finally resulting in augmented numbers of uMCs *in situ* [65]. Adoptive transfer of Tregs also was associated with elevated frequency of Tregs in decidua, thymus, and spleen of abortion mice [65]. According to the findings of this study, the interplay between Tregs and uterine mast cells could be related to the changes required for normal pregnancy development at the feto-maternal interface [65].

Toxoplasma gondii (T. gondii) is an intracellular parasite that can cause pregnancy complications such as abortion and stillbirth [72]. It has been indicated that toxoplasma gondii infection could reduce the percentage of CTLA-4^+ Tregs and PD-1^+ Tregs via enhancing apoptosis [73], which in turn may reduce the ratios of IL-10/IFN-γ and TGF-β/IFN-γ at the feto-maternal interface and in the spleen of pregnant mice [74]. Liu et al. indicated that pregnancy outcome of infected mice with T. gondii could be improved after the adoptive transfer of Tregs [24]. Mechanistically adoptive transfer of Tregs infected with T. gondii resulted in a reduction of abortion rates, placental hemorrhage, and an increase in the fetus weights compared to that of untreated infected mice [24]. The frequency of CTLA-4^+ Tregs , PD-1^+ Tregs and the ratios of IL-10/IFN-γ and TGF-β/IFN-γ increased in the infected group injected with Tregs from the fetal-maternal interface rather than spleen relative to untreated and infected controls [24]. Thus, the transfer

of Tregs from the fetal-maternal interface provides the balance between tolerant cytokines and inflammatory cytokines leading to an enhanced maternal tolerance.

Biological and pharmaceutical interventions to expand Tregs for spontaneous abortion

Various studies have attempted to develop innovative methods to increase the number of Tregs in peripheral blood [75-80]. Tregs are a small population of peripheral blood mononuclear cells, and it is hard to obtain enough Tregs for therapeutic purposes [81]. The main aim of expanding Tregs *ex vivo* is to provide a sufficient number of cells, modifying the Tregs-Teffector cells balance [82]. It has been shown that *ex vivo* expanded Tregs are more suppressive than freshly isolated cells [70]. The *ex vivo* expanded Tregs produce high levels of inhibitory cytokines, such as IL-10 and TGF-β, which can suppress the proliferation of effector T-cells [83, 84].

The effectiveness of TGF-β, Fingolimod [27, 85] and Trichostatin A [58] as the stimulators of Tregs expansion to prevent abortion in mouse models has been evaluated *in-vitro*. It has been hypothesized that Tregs among the $Thy1.1^{+}$ CFSE-labeled unsorted cells may expand by IL-2 and Flt-3 [86]. It is well known that *ex vivo* induction of Tregs with TGF- β can act like a vaccine that generates host suppressor cells with the potential to protect major histocompatibility complex (MHC)-mismatched organ grafts from rejection [87]. The previous study demonstrated that $CD4^{+}CD252^{-}$ T cells isolated from the spleens of pregnant CBA/J mice in the presence of TGF-β could be converted to $CD4^{+}CD25^{+}$ T cells [88]. The key characterization of TGF-β induced Tregs is the contact-dependent mechanism of action that was not affected by anti- TGF-β or anti-IL-10

[25]. This property distinguishes TGF-β -converted Tregs from Th3 and Type 1 regulatory T cells, the activities of which are solely dependent on soluble TGF-β and IL-10, respectively [25].

Two separated studies showed that the adoptive transfer of either freshly isolated Tregs or TGF-β (5 ng/ml) induced Tregs at the early stage of pregnancy increased the proportion of Tregs in the spleen and decidua, FOXP3 mRNA and protein levels, IL-10 and TGF- β levels as well as decreased IFN-γ levels in the decidua. These changes were associated with reduced rates of spontaneous abortion. Consequently, Tregs or TGF-β-induced Tregs could maintain immune tolerance during pregnancy.

In vivo treatment of mice with Fms-like tyrosine kinase 3 ligand (Flt3-L) results in a significant increase of DCs in all primary and secondary lymphoid tissues [89]. Administration of Flt3-L to mice and humans expands Tregs via a significant increase in DCs subsets in peripheral blood secondary lymphoid organs, suggesting that Flt3-L might be a novel therapeutic agent in autoimmune diseases [90]. It has been shown that the adoptive transfer of $Thy1.1^{+}$ CFSE-labeled unsorted cells from peripheral lymph nodes and spleen from BALB/c mice (donor Tregs represented 0.1% of splenocytes or lymph node cells [91] treated with low dose IL-2, for 10 consecutive days, starting 4 days before mating) and 4 subcutaneous injections of 10 mg of Flt-3-l (6 days before mating) was effective in the prevention of fetal loss in CBA/J mice that were mated with DBA/2 mice [86]. Therefore it is tempting to speculate that Tregs expansion by Flt3-L or low-dose IL-2 treatments prevented pregnancy loss in abortion-prone mice [86].

In mammals, CpG motifs that are considered as pathogen-associated molecular patterns recognized by Toll-like receptor-9 can cause adverse immune responses resulting in embryo loss or preterm birth [92]. One of the characteristic features of NOD mice is a lower frequency of

IL-10^+ Tregs and prone to abortion [93]. The abortion rate in NOD mice is remarkably higher when CpG challenge and anti-IL-10 injection is performed compared to WT mice showing a link between immune response mediated by CpG and immune tolerance mediated by Tregs and IL-10 at the feto-maternal interface [27]. In NOD mice, FTY720 known as Fingolimod (a promising immunosuppressant drug) effectively converted conventional $CD4^+CD25^-$ cells into $Foxp3^+CD4^+CD25^+$ cells (iTreg cells) *in vitro* and *in vivo* [27]. Transfusion of iTregs (2×10^6 cells from the spleen of NOD mice that induced by FTY720 into NOD pregnant mice challenged with CpG decreased the fetal resorption and preterm birth that was associated with increased decidual $FOXP3^+$ Tregs and IL-10^+ cell numbers compared to WT mice. These findings indicate that IL-10^+ Tregs function is critical when mammals are challenged by CpG to maintain a favorable feto-maternal microenvironment.

Unstable FOXP3 (as a master regulator of Tregs) gene expression may lead to disruption of a successful pregnancy via maternal tolerance breakdown [3]. Epigenetic modifications, including methylation or acetylation, may reduce or increase the FOXP3 gene expression, respectively [94]. The acetylation of FOXP3 histone can improve the stability of FOXP3 levels by preventing proteasomal degradation and by increase chromatin remodeling contributing to access for transcription factors [95, 96]. It has been shown that histone deacetylases inhibitors (HDACs) enhance gene expression by reducing histone-DNA and non-histone protein interactions that lead to increased FOXP3 histone acetylation and eventually increase FOXP3 gene expression[97]. It has been suggested that Trichostatin A (TSA) (known as an antifungal antibiotic) through a couple of mechanisms promote Tregs stability and function. Trichostatin A can increase FOXP3 acetylation levels through inhibiting class I, II, and IV histone deacetylases

resulting in improvement of FOXP3 gene expression [98, 99]. Furthermore, Trichostatin A promotes the immunosuppressive activity of Tregs in models of transplant through inducing Tregs conversion from CD4+CD25− T cells extracted from peripheral T cells to Tregs $CD4^{+}CD25^{+}FOXP3^{+}$ *in-vitro* [100, 101]. Interestingly, TSA-induced $CD25^{+}CD25^{+}$ T-cells expressed elevated FOXP3 levels that were comparable to those found in nTreg [101].

In a study by Wang et al. iTregs were injected into pregnant CBA/J mice mated with DBA/2J males on Day 1 and 4 of pregnancy, respectively. Surprisingly, transfer of TSA induced Tregs significantly reduced abortion rate and increased frequency of CD4+CD25+Foxp3+ Tregs and PD-1, CTLA-4 gene expressions as well as TGF-β and IL-10 levels in the spleens of miscarriage prone mice at either early stage of pregnancy or embryo implantation stage [97]. This suggests that the transfusion of Tregs treated with TSA at both the early stage of pregnancy and the embryo implantation stage was effective in promoting immunosuppressive function of Tregs which contributed to a reduction in fetal rejection [13, 26, 28, 65, 102]. It could be hypothesized that TSA-induced Tregs *in vitro* may have the greatest effect on maternal tolerance to prevent abortion relative to freshly isolated Tregs or Tregs expansion with other stimulators utilized *in-vitro [97]*.

The salient features of studies related to the adoptive transfer of Tregs and spontaneous abortion in mouse models have been shown in Table 1.

Challenges of adoptive transfer of Tregs in spontaneous abortion

Evaluation of any strategy with Tregs in the human reproductive system must be taken cautiously. The advantage of reproductive problem solving compared with the possibility of

harmful immune diseases must also be considered. Probable adverse effects of artificially reinforcing the maternal Tregs, including reduced protection from pathogenic microorganisms [103] or even diminished immune surveillance [104] against tumors need to be taken into account.

Another issue that needs attention is the appropriate dose and subsets of Tregs [105]. Key challenges for utilizing of Treg therapy in pregnancy are the diagnosis of Treg cell deficiency and determination of appropriate time of adoptive transfer of Tregs. To our knowledge, the investigation of Treg cell deficiency in the blood or endometrium of women with spontaneous abortion was not the primary endpoint of any of the studies. The establishment of a standardized concept of minimum necessary Treg markers will be a useful step (164). According to the mouse studies evaluated in this study, the effects of Tregs appear to be most critical at the time during the implantation and early placentation phase of pregnancy [13, 26, 28, 65, 102]. The timing would need to harmonize with hormone regulation throughout the menstrual cycle, and the probable impacts of estrogen and progesterone on controlling the expansion of the Tregs should be considered [106].

It is worthy of considering that the Tregs phenotypes, function and sensitivity to priming could be adversely affected by the factors that increase inflammation in women including chronic infection, smoking, diabetic and pre-diabetic conditions, obesity and microbiome dysbiosis [107, 108]. Vitamins and [109], deficiencies in micronutrients and microbiome disorders could also affect Treg cells. We have previously shown that VitD deficiency can cause a reduction in Tregs frequency [57] and signature gene expressions of these cells such as GITR and FOXP3[58].

Treatment of the deficiencies listed may be useful for improving Tregs activity in the reproductive system, as demonstrated for some other immune disorders [110].

Concluding remarks and future directions

Tregs are recognized as a pivotal subset of immune cells with immune-modulatory properties which could play a key role in maintaining maternal tolerance. A large body of literature indicated that any deregulation of the frequency or function of Tregs could contribute to spontaneous abortion. Researches reviewed in this study led us to postulate that Tregs isolation from pregnant women, *ex vivo* expansion and autologous transfer could be a promising strategy to improve the outcome of pregnancy in women with spontaneous abortions. Transfer of Tregs appears to be most important in the implantation phase and early stages of pregnancy from the feto-maternal interface, which may be necessary for the maintenance of the later stages of pregnancy and reduced abortion rates. These points should be considered in clinical approaches.

Evaluation of any strategy for the Tregs in the human reproductive system must consider a very careful approach and be predicated on appropriate frameworks of clinical trials. Probable adverse effects of artificially strengthen maternal Tregs, including reduced protection from pathogenic microorganisms or even diminished immune surveillance against tumors needs to be considered.

Briefly, the adoptive transfer of Tregs via modulation of pro-inflammatory and anti-inflammatory cytokine responses and the enhancement of angiogenesis could inhibit spontaneous abortion.

Evidence-of-concept studies in abortion prone mice already showed the utility of biological and pharmaceutical agents including TGF-β, Fingolimod, and Trichostatin A to boost numbers of Tregs and stability. Other potentially biological agents to induce Tregs cell-mediated tolerance that their effect on the ex-vivo expansion of Tregs in spontaneous abortion has not been evaluated and is worthy of examination including CSF3 (clinically used to improve embryo implantation and placentation [111], Flt3-L and low dose IL-2[86], IL-10 [112] and several micro RNAs [113] progesterone [114, 115] (mediates suppression of the T cell response but whether the impacts on Tregs is unknown), humanized antibodies against T cell markers such as anti-CD3, anti-CD52, and anti-CD45RO/RA and cytokine specific monoclonal antibodies such as anti-TNF-α (which restore immune tolerance by stabilizing Tregs) [116].

Table.1: Characterization of studies related to the adoptive transfer of Tregs and spontaneous abortion in mouse models.

The studied groups of mouse models	The source of isolated Tregs	Purification (%) of isolated Tregs	Expansion of Tregs *in vitro*	Number of transferred Tregs/rout of transfusion/day of transfusion	Outcome after the adoptive transfer of Tregs	ref
Control group: pregnant CBA/J females mated with BALB/c Abortion groups: (DBA/2J-mated CBA/J) 1. control abortion group that were given PBS 2.abortion group that received Tregs from non-pregnant CBA/J virgin females 3. Abortion group that received Tregs from 14-day BALB/c pregnant CBA/J females	Spleen and thymus cells from non pregnant or 14-day normal pregnant mice	96 -98%	-	2×10^5/ i.v/ 0 to 2 of pregnancy	The reduction of proliferation IFN-γ^+ Th1 cells and secretion of IFN-γ, a significant up-regulation of IL-10 expression in decidua and placenta of abortion-prone mice, A significant reduction in the abortion rate in abortion prone mice	[13]
Control group: CBA/J mice mated with BALB/c Abortion groups: 1.mice received freshly isolated Tregs on day 4 of pregnancy (implantation stage) 2. mice received freshly isolated Tregs on day1 (at an early stage) of pregnancy 3.mice received freshly isolated Tregs on day 4 or 1 of pregnancy 4.mice received *in vitro* expanded Tregs (for 8 days) on day 1 and 4 of pregnancy	Splenic Tregs from non-pregnant CBA/J mice	≥93	Tregs were stimulated with anti-mouse CD3 antibody and anti-mouse CD28 antibody in the presence of recombinant mouse IL-2 for 8 days	1.5×10^5 / i.v/ on day 1, 4 or on both day of 1 and 4 days of pregnancy	Particularly *in vitro* expanded Tregs, at an early stage of pregnancy caused a reduction in abortion rates that was associated with increased the ratios of serum IL-10/ IFN-γ or TGF-β1/ IFN-γ in the mouse model of spontaneous abortion	[26]
Control group: CBA/J mice mated with BALB/c	Decidua of pregnant	94-97%	Tregs were stimulated	2×10^5/	The transfer of expanded *in vitro* Tregs	[28]

Abortion groups: 1. CBA/J mice mated with BALB/c mice received rIL-17 (10 μg/mouse) on Day 1 of pregnancy. 2. CBA/J mice mated with BALB/c mice received Tregs 2 days before mating 3. CBA/J mice mated with BALB/c mice received Tregs on Day 7 of pregnancy	CBA/J × BALB/c mice		with immobilized anti-mouse CD3 antibody and anti-mouse CD28 antibody in the presence of recombinant mouse IL-2 for 3-5 days	i.v/before mating, on 1 day and 7 days of pregnancy	from normal pregnant mice before mating significantly increased IL-10 and TGF-β level in decidua and reduced the abortion numbers	
Control group: CBA/J mice mated with BALB/c Abortion group: CBA/J mice mated with DBA-2J	Spleens and lymph nodes of normal pregnant mice	Not mentioned	-	2×10^5 cells/i.v / day 0 of pregnancy	The adoptive transfer of Tregs was associated with a diminished abortion rate and a rise in the proportion of uMCs in the placenta, in the oviduct and the splenic tissue as well as a decrease in sFlt-1 levels. These alternations helped to improve the remodeling spiral artery and increased placenta size.	[65]
1. Control group: C57BL/6 (female) × BALB/C (male) pregnancy group 2. pregnancy group infected with T.gondii 3. pregnancy infected group that received Tregs from the spleen 4. pregnancy infected group infected mice that received Tregs from the placenta and the uterine	Spleen cells or placenta and uterine cells from normal pregnant mice	>95%/	-	2×10^5 /i.v/ day 8 of pregnancy	Transfer of Tregs from the fetal-maternal interface and the spleen of pregnant mice infected with T. gondii) resulted in reducing of abortion rates, placental hemorrhage and increasing the frequency of CTLA-4^+ Tregs, PD-1^+ Tregs and the ratios of IL-10/IFN-γ and TGF-β/IFN-γ in the infected group injected Tregs from the	[24]

					fetal-maternal interface rather than spleen relative to untreated and infected controls	
Control group: CBA/J mice mated with BALB/c Abortion groups: 1. CBA/J mice mated with DBA/2 mice (abortion model without treatment); 2. CBA/J mice mated with DBA/2 mice and injected $CD4^+CD25^+FOXP3^+$ Treg cells on Day 1 of pregnancy 3. CBA/J mice mated with DBA/2 mice and injected TGF-β1-induced Tregs on Day 1 of pregnancy 4. CBA/J mice mated with DBA/2 mice and injected $CD4^+CD25^-$ T cells on Day 1 of pregnancy.	Splenic cells from pregnant CBA/J mice mated with BALB/c mice	99.05	$CD4^+CD25^-$ T cells were stimulated with anti-CD3, anti-CD28, TGF-β1 and IL-2 for 5 days		The adoptive transfer of either fresh nTregs or TGF-β induced Tregs at the early stage of pregnancy increased the proportion of Tregs in the spleen and decidua, FOXP3 protein and mRNA levels in the decidua, IL-10 and TGF- β levels as well as decreased IFN-γ levels	[25, 102]
Control group: Female BALB/c (female) mated with male C57BL/6 Abortion group:NOD mice mated with C57BL/6 received CpG1826 at doses 15 to 500 μg per dam on 6.5 days of the gestation day preterm birth group: NOD mice mated with C57BL/6 received CpG1826 at doses 15 to 500 μg per dam on 14.5 days of the gestation day	Splenic CD4+ from virgin Female BALB/c and NOD mice	95-97%	Treg cell $CD4^+CD25^-$ T cells cultured in the presence of anti-CD3 Ab, rIL-2 and FTY720 for 6 days	2×106/i.v/8 hours after CPG challenging	Treg Therapy decreased the fetal resorption and preterm birth that was associated with increased decidual $FOXP3^+$ Tregs and $IL\text{-}10^+$ cell number compared to WT mice	[27]

Control group: CBA/J mice mated with BALB/c CBA/J × DBA/2J matings were used as the miscarriage-prone model Abortion groups: 1.Spontaneous abortion group without treatment 2. Injection of iTreg with TSA treatment on day1 of pregnancy 3. Injection of iTreg with TSA treatment on day4 of pregnancy 4. Injection of iTreg without TSA treatment post-TCR stimulation on day1 of pregnancy 5. Injection of freshly isolated CD4+CD25- T cells on day1 of pregnancy 6. Injection of freshly isolated CD4+CD25+ Treg on day1 of pregnancy	Isolated CD4+CD25 − T-cells from the spleen of non-pregnant CBA/J mice	>92%	$CD4^{+}CD25^{-}$ T-cells were stimulated with immobilized anti-mouse CD3antibody, anti-mouse CD28 antibody and recombinant mouse IL-2 for 72 h. further were cultured in the presence or absence of TSA	1×10^{6} /i.v	Transfusion of TSA induced Tregs significantly increased the population of $CD4^{+}CD25^{+}Foxp3^{+}$ induced Tregs expressed high levels of PD-1 and CTLA-4, and secreted high levels of TGF-β and IL-10	[97]

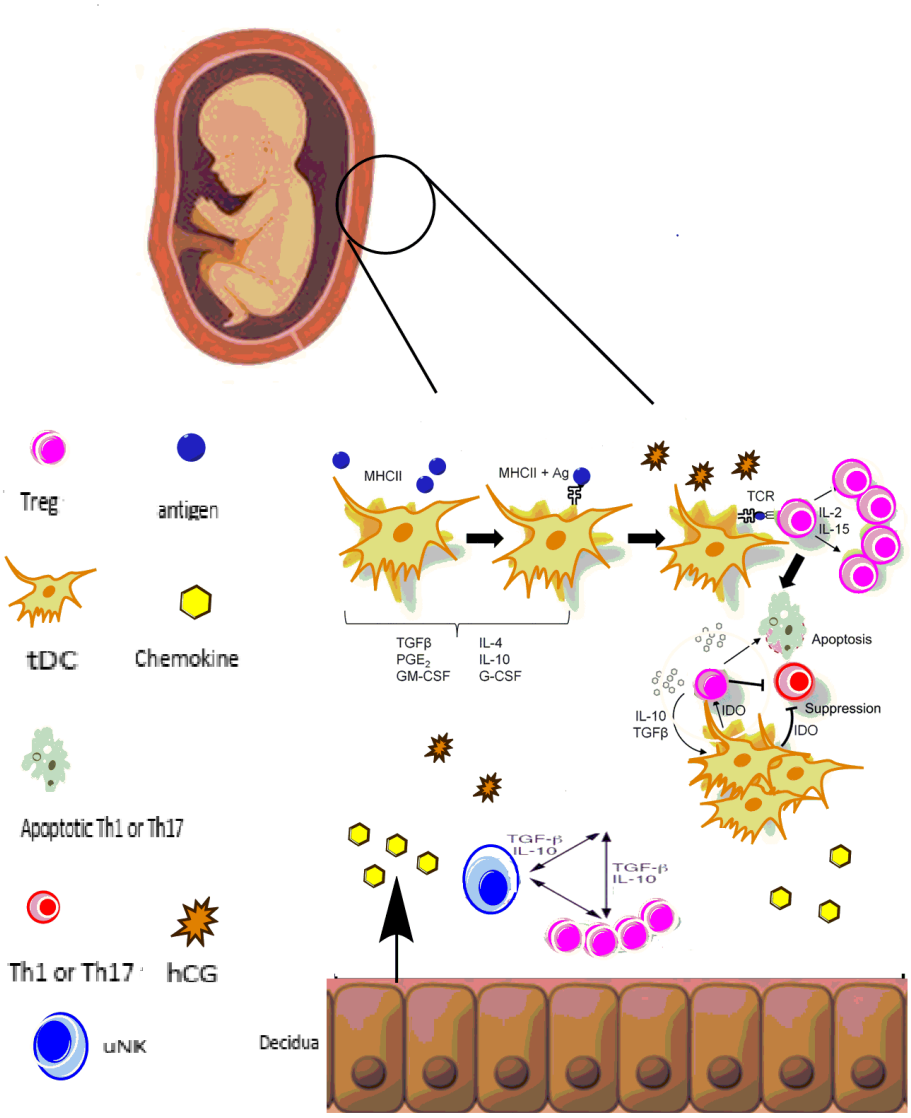

Fig. 1 A schematic illustration of the Tregs activation and action at the feto-maternal interface. After parental antigen uptake and processing, DCs present antigen fragments on their surface in association with class II MHC molecules. The tolerogenic DCs activate the Treg cells that express cognate TCRs and then proliferate. Treg cells also recruited by chemokines and hCG to the pregnancy microenvironment. Treg cells secrete IL10 and TGFb and induce IDO expression in local DCs to further activate and maintain suppressive function in Treg cells, inhibit Th1 and Th17 cell proliferation, modulate decidua uNKs and induce Th1 and Th17 cell apoptosis. Ag = antigen; DC = dendritic cell, G-CSF = granulocyte colony-stimulating factor; GMCSF = granulocyte-macrophage colony-stimulating factor; hCG: human chorionic gonadotropin IDO = indoleamine 2,3-dioxygenase; IL = interleukin; MHC = major histocompatibility complex; TCRs: T-cell receptor; Th1=T helper 1;Th17=T helper 17; TGF-β = transforming growth factor; uNK= uterine NK

Abbreviations

CTLA-4: Cytotoxic T-lymphocyte-associated protein 4

CSF3: Colony Stimulating Factor 3

DCs: Dendritic cells

Flt3-L: Fms-related tyrosine kinase 3 ligand

FOXP3: forkhead box P3

GITR: glucocorticoid-induced tumor necrosis factor receptor

HCG: human chorionic gonadotropin

HDACs: Histone deacetylases inhibitors

IL-4: interleukin-4

IL-10: interleukin-10

IL-17: interleukin-17

IFN-γ: Interferon-gamma

iTregs. inducible regulatory T cells

i.v: intravenously

MHC: Major histocompatibility complex

mSCF: murine stem cell factor

NK: natural killer

NKT: natural killer T cells

NOD : none obese diabetic

nTregs: natural regulatory T cells

PAMPs: pathogen-associated molecular patterns

PD-1: programmed cell death 1

TCR: T-cell receptor

Th1: T helper-1

Th3: T helper-3

Th2: T helper-2

Th17: T helper-17

TLR9: Toll-like receptor-9

Tregs: regulatory T cells

TGF-β: transforming growth factor-beta

TNF-α: tumor necrosis factor-alpha

TSA: Trichostatin A

uMCs: uterine mast cells

VEGFR: Vascular endothelial cell growth factor receptor

WT: wild type

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