Journal of Reproductive Immunology 131 (2019) 30–35

Contents lists available at ScienceDirect

Journal of Reproductive Immunology

journal homepage: www.elsevier.com/locate/jri

Effect of adoptive transfer of $CD4^+CD25^+Foxp3^+$ Treg induced by trichostatin A on the prevention of spontaneous abortion

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ARTICLE INFO

Keywords:
Unexplained recurrent spontaneous abortion
Histone modification
Trichostatin A
$CD4^+CD25^+Foxp3^+$ Treg
Adoptive transfer
Embryo resorption rate

ABSTRACT

Objective: To investigate whether epigenetic modification of $CD4^+CD25^-$ T-cells in vitro can make up for the inadequacy of $CD4^+CD25^+Foxp3^+$ Treg in animal model of spontaneous abortion and prevent immune response-mediated spontaneous abortion.

Methods: Trichostatin A (TSA) was applied to inhibit histone deacetylases (HDACs) and thereby to epigenetically modify the special location of Foxp3 gene in $CD4^+CD25^-$ T-cells of CBA/J mice. The expressions of CD25, Foxp3, CTLA-4 and PD-1 of $CD4^+$ T cells isolated from spleen of mice were characterized by flow cytometric analysis. Concentrations of transforming growth factor- β (TGF-β) and IL-10 in the supernatants of cultured Treg were measured using ELISA. The purified $CD4^+$ T cells treated with different reagents were injected into pregnant CBA/J mice mated with DBA/2J males on Day 1 and 4 of pregnancy, respectively. The embryo resorption rate was assessed on Day 14 of pregnancy.

Results: TSA treatment significantly increased the population of $CD4^+CD25^+Foxp3^+$ iTreg. Those TSA induced Treg expressed high levels of PD-1 and CTLA-4, and secreted high levels of TGF-β and IL-10. Adoptive transfer of those iTreg at both early stage and implantation of stage of pregnancy significantly increased population of $CD4^+CD25^+Foxp3^+$ Treg in spleens of recipient miscarriage prone mice and significantly reduced resorption in those mice.

Conclusion: Epigenetic regulation of Foxp3 can generate functional regulatory T-cells. Adoptive transfer of TSA-induced $CD4^+CD25^+Foxp3^+$ Treg at an early stage of pregnancy can induce maternal-fetal immune tolerance and reduce embryo resorption in miscarriage prone mice.

1. Introduction

Unexplained recurrent spontaneous abortion (URSA), which is defined as the loss of two or more consecutive pregnancies before the 20th week of gestation – with the exception of many known causes (anatomic, infectious, hormonal, immunological, and genetic), occurs in approximately 0.4%–2.5% of reproductive-age women (Wang et al., 2010). Feto- maternal immune tolerance supports normal pregnancy (Bonney and Brown, 2014). On the other hand, immunological dysfunction has been proposed to cause URSA. For example, it has been shown that the fetal semi- allograft is attacked by alloreactive $CD4^+$ effector T-cells or $CD8^+$ cytotoxic T-cells from the mother (Saito et al., 2005).

Regulatory T cell (Treg) is $CD4^+CD25^+$ T cell expressing the forkhead/winged helix transcription factor protein-3 (Foxp3) as a specific and functional marker (Davidson et al., 2007). Natural $CD4^+CD25^+Foxp3^+$ Treg (nTreg) cells represent 1–3% of total $CD4^+$ T-cells of spleen or peripheral blood in human and 5–10% in mice. It has been reported that there is a negative correlation between the proportion of Treg and the probability of URSA (Zenclussen et al., 2005). Increasing Treg has been postulated to prevent URSA (Arruvito et al., 2007). Numerous researchers have attempted to establish novel methods to expand the $CD4^+CD25^+Foxp3^+$ Treg population in peripheral blood (Davidson et al., 2007). The co-stimulation with anti-CD3

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https://doi.org/10.1016/j.jri.2018.12.002
Received 19 August 2016; Received in revised form 24 September 2018; Accepted 25 December 2018

mAb and anti-CD28 mAb coupled with TGF-β or IL-2 treatment may induce CD25 and Foxp3 expression in $CD4^+CD25^-$ T-cells, but the resulting $CD4^+CD25^+Foxp3^+$ Treg cannot exihibit stable phenotype and suppressive function (Schmidt et al., 2016).

Previous work has suggested that epigenetic modification can result in permanent and stable expression of CD25 and Foxp3, which induces suppressive characteristics of Treg and promotes the conversion of $CD4^+CD25^-$ T-cells to nTreg (Kwon et al., 2012). In recent years, increasing amount of evidence has also shown that HDACs inhibitor TSA allows Foxp3 transcription (acetylation) by up-regulating chromatin remodeling (Moon et al., 2009) and that treatment with TSA may enhance Foxp3 gene expression and the immunosuppressive effect of Treg in models of transplant (Tang et al., 2013). Therefore, increasing Foxp3 acetylation levels is postulated as an effective approach to generate induced Treg from peripheral T cells.

In the present study we set forth to determine whether inhibition of histone deacetylation could generate functional $CD4^+CD25^+Foxp3^+$ Treg and whether adoptive transfer of the induced Treg could reduce miscarriage rate in CBA/*J* mice mated with DBA/2 J mice (a miscarriage-prone model).

2. Materials and methods

2.1. Mice and grouping

Eight-week-old inbred female CBA/J (H^{2k}), age-matched male BALB/c (H^{2d}) and male DBA/2 J (H^{2d}) mice weighing 25–30 g were purchased from Beijing HFK Bioscience Co. Ltd. (Beijing, China). All mice were maintained in a specific pathogen-free animal facility. They were housed under a 12 L:12 D cycle and an ambient temperature of 22 ± 1 °C. All experimental protocols and animal care procedures used in this investigation were approved by the Ethics Committee of Shanxi Datong University. Females were caged individually overnight with a fertile male for mating, after which they were examined twice a day for signs of pregnancy. The day of appearance of postcoital vaginal plug was designated as Day 0 of pregnancy.

CBA/J × BALB/c matings were used as the normal control pregnant model. CBA/J × DBA/2J matings were used as the miscarriage-prone model, which were further divided into 6 groups, the characteristics of grouping are shown in Table 1. Bodyweight of mice was determined on the day of grouping and injection, respectively, there was no significant difference in body weight among CBA/J mice of experimental groups 1–7.

2.2. Isolation and culture of mouse T-cells

The non-pregnant CBA/J mice were used as negative control and sacrificed. Single splenic cell suspensions were prepared. $CD4^+$ T-cells were negatively selected by magnetic-activated cell sorting (MACS) according to the manufacturer's instructions (Miltenyi Biotec, Germany), and cells were incubated with anti-CD25 micro-beads. $CD4^+CD25^+$ T-cells and $CD4^+CD25^-$ T-cells were separated from $CD4^+$ T-cells by a positive selection method. The purity was then confirmed by flow cytometric analysis.

2.3. TSA treatment

The isolated $CD4^+CD25^-$ T-cells from non-pregnant CBA/J mice were cultured in 96-well culture dishes at a density of 1×10^6 cells/mL. TCR stimulation was performed by immobilized anti-mouse CD3 antibody (5 μg/mL, eBioscience), anti-mouse CD28 antibody (3 μg/mL, eBioscience), and recombinant mouse IL-2 (500 U/mL) in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, 1% penicillin/streptomycin, 1% glutamine at 37 °C with 5% CO_2 (Tai et al., 2005). After 72 h of stimulation, $CD4^+CD25^-$ T-cells were cultured further in the presence or absence of TSA at concentrations in the range of 1–1000 nM (Sigma) at 37 °C for 72 h. TSA was dissolved in DMSO solution serving as vehicle, TSA was replaced with DMSO in the control cells. The media were replaced with new ones every three days.

2.4. Adoptive transfer of $CD4^+CD25^+Foxp3^+$ Treg

The freshly isolated $CD4^+CD25^-$ T-cells, nTreg, or TSA- induced Treg in vitro were washed twice with cold PBS, counted, diluted to 1×10^6 cells/mL in PBS. Two hundred μL of dilution was injected into the pregnant CBA/J mice from groups 3 to 7 on day 1 or 4 of pregnancy, respectively, via tail vein. The mice injected with PBS alone were used as negative controls.

2.5. Flow cytometry for Treg

Female CBA/J mice from groups 1 to 7 were sacrificed on Day 14 of pregnancy, and single splenic cell suspensions were prepared. The isolated Treg were characterized by flow cytometry analysis using FITC anti-mouse CD4, and PE anti-mouse CD25 at 4 °C for 30 min. After permeabilization, the cells were stained with PE-cy5 anti-mouse Foxp3 on a BD Calibur flow cytometer using Cellquest Pro software (Becton Dickson, USA), the expression of Foxp3 in Treg was measured. Cultured cells were harvested and washed with cold PBS. The mean fluorescence intensity was analyzed immediately by flow cytometry after staining with FITC-anti-CD4, PE-cy5-anti-CTLA-4, and PE-anti-PD-1 following the protocol recommended by the manufacturer. All mAbs were purchased from eBioscience (San Diego, USA). At least 10, 000 live cells were acquired for each analysis, and statistical analysis was performed using the isotype- matched controls as a reference.

2.6. Determination of cytokine levels

The cell supernatants were collected after TCR stimulation and/or TSA treatment. Concentrations of TGF-β and IL-10 in TSA- treated supernatants were determined by ELISA using specific kits (BioSource, USA), according to the instructions from the manufacturer.

2.7. Fetal resorption

On Day 14 of pregnancy, the pregnant mice from groups 1 to 7 were sacrificed by cervical dislocation, their uteri were removed and the implantation sites were examined. The resorbed embryos were identified by their small size and necrotic hemorrhagic appearance compared

Table 1
The characteristics of different groups.

Group	Number	Mating Combination	Treatment
Group 1	10	CBA/J × BALB/c	Normal pregnancy control
Group 2	10	CBA/J × DBA/2J	Spontaneous abortion group without treatment
Group 3	10	CBA/J × DBA/2J	Injection of iTreg with TSA treatment on day1 of pregnancy
Group 4	10	CBA/J × DBA/2J	Injection of iTreg with TSA treatment on day4 of pregnancy
Group 5	10	CBA/J × DBA/2J	Injection of iTreg without TSA treatment post-TCR stimulation on day1 of pregnancy
Group 6	10	CBA/J × DBA/2J	Injection of freshly isolated $CD4^+CD25^-$ T cells on day1 of pregnancy
Group 7	10	CBA/J × DBA/2J	Injection of freshly isolated $CD4^+CD25^+$ Treg on day1 of pregnancy

to normal embryos. The percentage of fetal resorption was calculated according to the following formula: R = Re/(Re + F), where Re is the number of resorbed embryos and F denotes the number of surviving embryos (Jaiswal et al., 2011).

2.8. Statistical analysis

All the experimental data were analyzed with SPSS software 17.0, and the values were presented as mean ± SD. Independent sample *t*-tests were used to compare the percentage of Treg in $CD4^+$ T-cells among 7 groups, and estimate the expression of CTLA-4, PD-1 of $CD4^+$ T cells with TSA treatment. The differences in the levels of TGF-β and IL-10 among the groups were analyzed by the non-parametric Mann-Whitney *U* test. Comparisons were performed by chi-square test for the embryonic resorption rates among the groups. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Treg population in T cells in mice with normal or URSA pregnancy

It has been shown that in both humans and mice the portion of $CD4^+CD25^+$ T-cells to total $CD4^+$ T-cells is lower in pregnancy from miscarriage-prone mating compared to normal pregnancy (Zenclussen et al., 2005; Lee et al., 2011). To assess the percentage of $CD4^+CD25^+$ T-cells in pregnant CBA/J mice mated with BALB/c or DBA/2J mice, we sacrificed pregnant mice on Day 14 of gestation and isolated T-cells from spleens. We analyzed splenic $CD4^+CD25^+$ T-cells by flow cytometry. As shown in Table 2, the percentage of $CD25^+$ cell in URSA group (group 2) is significantly lower than that in normal pregnancy group (group 1). Since Foxp3 is a master regulatory factor and specific marker of Treg, we further determined the expression of intracellular Foxp3 in the $CD4^+$ T-cells. Compared to control group, the percentage of $Foxp3^+$ T-cells reduced significantly in the URSA group (Table 2). The percentage of both $CD25^+$ and $Foxp3^+$ cells in URSA group was only half of that of the normal control group (Table 2).

3.2. TSA induction of $CD4^+CD25^+Foxp3^+$ Treg

As mentioned in Introduction, inhibition of HDACs by TSA induces Foxp3 transcription (Zhang et al., 2012). To investigate whether TSA has any effects on population of $CD4^+CD25^+Foxp3^+$ Treg, we isolated $CD4^+CD25^-$ T-cells and treated them with various concentrations (1–1000 nM) of TSA for different durations to find optimal condition for induction of CD25 and Foxp3. The expression of both CD25 and Foxp3 increased as the concentration of TSA increased from 1 to 100 nM and decreased when the concentration was raised to 1000 nM (Fig. 1). We also determined that the optimal duration for Treg induction was 72 h. Overall the levels of CD25 and Foxp3 were significantly higher in cells treated with TSA compared to DMSO following TCR activation ($P < 0.01$, Fig. 2). TSA- induced $CD4^+CD25^+$ T-cells expressed high levels of Foxp3, which were similar to the levels detected in nTreg, and significantly higher compared with $CD4^+CD25^-$ T-cells (Fig. 2).

Furthermore, to characterize the function of TSA- induced Treg, using flow cytometry and ELISA, we evaluated the expression of PD-1, CTLA-4, TGF-β and IL-10 in the cultured cells. TCR stimulation alone increased the percentages of $CD4^+PD\text{-}1^+$ and $CD4^+CTLA\text{-}4^+$ T cells by 2 fold, increased TGF-β expression by 3 fold and increased IL-10 expression by almost 6 fold. The combination of TCR stimulation and TSA treatment resulted in more than 2 fold increase in percentage of $CD4^+PD\text{-}1^+$ T cells population and more than 3 fold increase in percentage of $CD4^+CTLA\text{-}4^+$ T cells. More impressively, the combination of TCR stimulation and TSA treatment caused 6–10 fold increase in TGF-β and IL-10 expression (Table 3). These increases in pro-growth factors were assumed to enhance the ability of Treg cells to support pregnancy and prevent URSA.

Table 2
The percentage of $CD25^+$ and/or $Foxp3^+$ T-cells in $CD4^+$ T-cells among 7 groups.

Group	$CD4^+CD25^+$ (%)	$CD4^+Foxp3^+$ (%)	$CD25^+Foxp3^+$ (%)
Group 1	14.69 ± 1.95**,***	13.97 ± 2.05**,***	12.85 ± 1.97**,***
Group 2	8.21 ± 2.01*	7.66 ± 2.05*	6.60 ± 1.99*
Group 3	11.70 ± 1.80**	11.01 ± 1.46**,***	10.09 ± 1.56**,***
Group 4	12.22 ± 1.82**,***	11.43 ± 1.99**,***	10.56 ± 1.98**,***
Group 5	10.30 ± 1.08*	9.70 ± 1.22*	8.62 ± 1.08*
Group 6	9.48 ± 1.35*	8.84 ± 1.37*	7.82 ± 1.37*
Group 7	12.29 ± 1.45**,***	11.56 ± 1.32**,***	10.69 ± 1.29**,***

* $P < 0.01$ vs. the control with normal pregnancy (group 1).
** $P < 0.01$ vs. the spontaneous abortion group (group 2).
*** $P < 0.05$ vs. the group with TCR stimulation only (group 5).

3.3. Adoptive transfer of iTreg decreased fetal resorption rates

Since iTreg have similar characteristics of nTreg and express high levels of pro-growth factors in vitro, we next set forth to determine whether these iTreg could prevent URSA in vivo. We isolated $CD4^+CD25^-$ T cells from non-pregnant CBA/J mice and treated them with or without anti- TCR and/or TSA for optimal duration and selected $CD4^+CD25^+Foxp3^+$ T cells from treated cells. We also isolated $CD4^+CD25^+$ T cells from non-pregnant CBA/J mice. The phenotypic purity of the freshly isolated T-cells reached greater than 92% (Fig. 1). We then injected those cells into pregnant CBA/J mice mated with DBA/2 J (miscarriage-prone) mice via tail vein. The treatment design is listed in Table 1. On Day 14, we sacrificed mice, isolated T cells from their spleens and assessed CD25 and Foxp3 expression levels. Transfer of $CD4^+CD25^-$ T cells resulted in slight and insignificant increases in percentages of $CD25^+$, $Foxp3^+$ and $CD25^+Foxp3^+$ cells (Table 2). Transfer of iTreg treated with only anti- TCR did not cause significant increase, either. Transfer of iTreg treated with both anti- TCR and TSA, however, resulted in significant increases in percentages of $CD25^+$, $Foxp3^+$ and $CD25^+Foxp3^+$ cells. These increases were slightly greater when those T cells were injected on Day 4 of pregnancy than on Day 1. Among all groups, the greatest increases were caused by injection of nTreg. The percentages of $CD25^+$, $Foxp3^+$ and $CD25^+Foxp3^+$ T cells in those pregnant CBA/J (miscarriage-prone) mice mated with DBA/2J mice were close to the percentages in normal pregnant mice (Table 2).

With a separate set of mice, we examined their pregnancy. Pregnant mice were sacrificed on Day 14 and the percentages of embryo resorption and fetus survival were assessed. As shown in Table 4, the embryo resorption rate in miscarriage-prone mice (group 2) was 3-fold higher than that in normal control mice (group 1) ($P < 0.01$), and the number of survival fetuses was lower in group 2 than in group 1, demonstrating that mating CBA/J mice with DBA/2J did result in higher rate of abortion. We treated these miscarriage-prone mice with injection of T cells at different stages of pregnancy. While transfer of $CD4^+CD25^-$ T cells (group 6) and PBS (negative control, Resorption rate: 21.43%) did not have significant effects on fetus survival, adoptive transfer of TSA- induced Treg significantly improved the pregnancy outcome of CBA/J × DBA/2J mating. Transfer of iTreg at either early stage of pregnancy (Day 1, group 3) or embryo implantation stage (Day 4, group 4) significantly reduced resorption rate by about 50% and increased fetus survival rate, although the effects were slightly greater when iTreg was injected at day 1 than day 4. Transfer of iTreg with TCR stimulation but without TSA treatment (group 5) was not as effective as transfer of iTreg with both TCR stimulation and TSA treatment. Among all treatments, transfer of $CD4^+CD25^+$ nTreg (group 7) exerted the greatest improvement of the pregnancy outcome, reducing the resorption rate to the level close to that of normal pregnancy and increasing the fetus survival rate to the level close to that of normal pregnancy. These effects of Treg on pregnancy (Table 4) positively correlate to

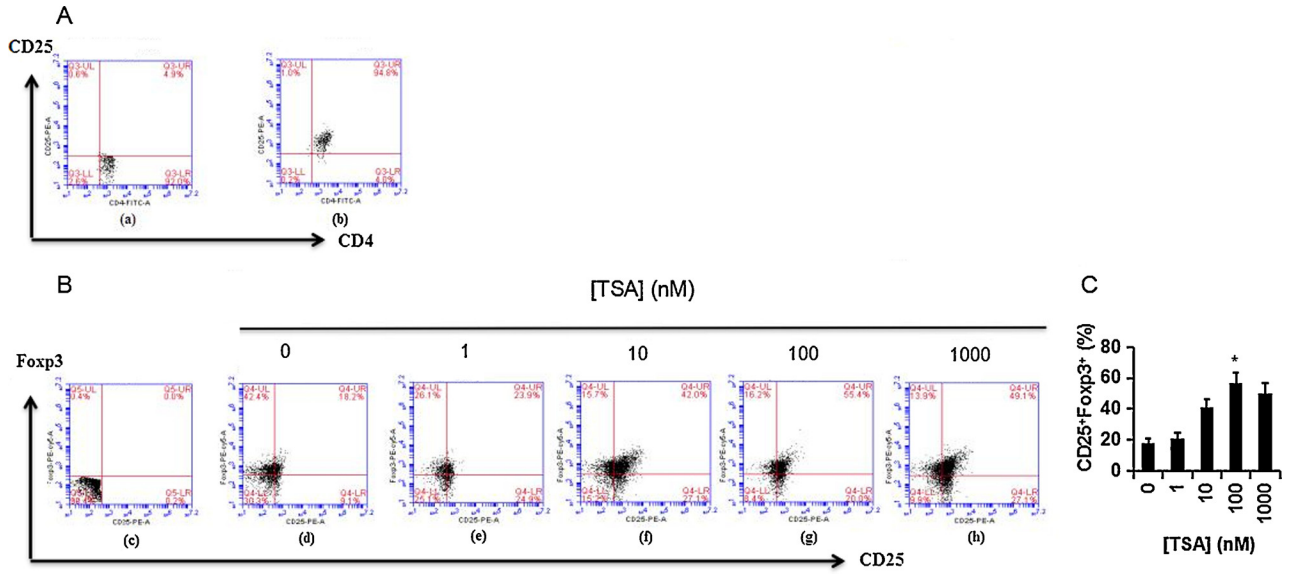

Fig. 1. Flow cytometry plots before and after TSA treatment. (A) Population of $CD4^+CD25^-$ T cells (a) and $CD4^+CD25^+$ T cells (b) after MACS isolation; (B) Representative FACS profiles comparing $CD4^+CD25^+Foxp3^+$ populations treated with different concentrations of TSA for 72 h and stimulated with anti-CD3 mAb, anti-CD28 mAb, and IL-2. The isotype control (c) was used to adjust the cross gate; and (C) The percentage of $CD25^+Foxp3^+$ Treg in $CD4^+$ T-cells after treatment with different concentrations of TSA, Values = mean ± SD (n = 10). *p < 0.05 (vs. other groups).

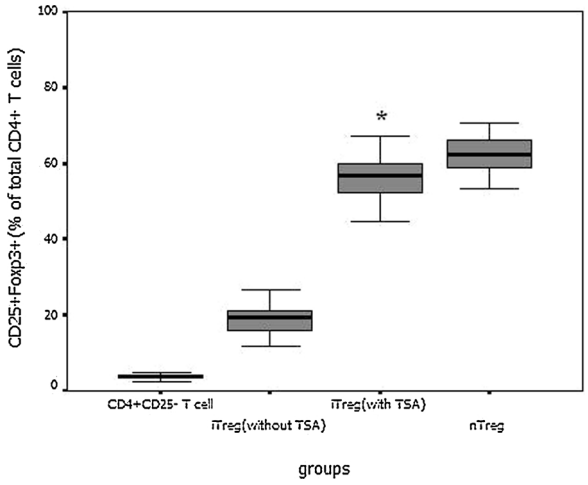

Fig. 2. The proportion of $CD25^+Foxp3^+$ T-cells in total $CD4^+$ T-cells. The upper and lower whiskers represent the maximum and minimum, respectively. The upper and lower limits of the boxes represent the 75th and 25th percentiles, respectively. The median is indicated by the line in each box. The median proportions were 3.7% (range: 3.32%–4.07%), 19.35% (range: 16.96%–20.82%), 56.75% (range: 53.01%–59.61%), 62.4% (range: 60.07%–64.69%) in $CD4^+CD25^-$ T-cell group, iTreg group (DMSO-only-treated following TCR activation), iTreg group (TSA-treated following TCR activation), $CD4^+CD25^+$ nTreg group, respectively, with statistically significant difference (*p < 0.05 vs. other groups).

Table 4
Comparisons of fetal resorption rates in pregnant CBA/J mice.

Group	Number of mice	Surviving fetuses	Resorbed fetuses	Resorption rate (%)
Group 1	10	83	6	6.74*,**
Group 2	10	62	18	22.50**
Group 3	10	75	8	9.64*,**
Group 4	10	71	10	12.35*
Group 5	10	70	13	15.66*
Group 6	10	67	15	18.29
Group 7	10	78	7	8.97*,**

* P < 0.01 vs. spontaneous abortion group (Group 2).
** P < 0.01 vs. The group injected with iTreg with TCR stimulation alone (Group 5).

those on percentages of $CD25^+Foxp3^+$ T cells (Table 2).

4. Discussion

It has been postulated that the recurrent pregnancy losses may be due to the failure of fetal-maternal immunologic tolerance. Treg (expressing CD4, CD25 and Foxp3) supports normal pregnancy and URSA has been reported to be negatively correlated to the population of $CD4^+CD25^+Foxp3^+$ Treg (Chen et al., 2013; Arruvito et al., 2007). In this study, we used female CBA/J × male DBA/2J mating as the miscarriage-prone model, which shares many features with human URSA and has been widely used for studies of immuno-mediated spontaneous fetal loss (Zenclussen et al., 2005; Bonney and Brown, 2014; Chen et al., 2013). It has been reported previously that the incidence of fetal

Table 3
The in vitro characteristics and functions of Treg cells.

Condition	$CD4^+PD\text{-}1^+$ (%)	$CD4^+CTLA\text{-}4^+$ (%)	TGF-β (pg/ml)	IL-10 (pg/ml)
No TCR stimulation nor TSA treatment	5.16 ± 1.12**	2.86 ± 0.59**	41.77 ± 21.87**	4.12 ± 1.21**
TCR stimulation alone	8.32 ± 1.05*	6.03 ± 0.84*	124.48 ± 34.62*	23.55 ± 14.60*
TCR stimulation + TSA treatment	12.43 ± 1.60*,**	9.66 ± 0.94*,**	235.99 ± 51.26*,**	41.88 ± 23.00*,**

* P < 0.01 vs. the control without stimulation or treatment.
** P < 0.01 vs. the group with TCR stimulation alone.

resorption in this model ranges from 20% to 40% (Redecha et al., 2009). In accord to the literature, the embryo loss rate in this study was 22.50%, which was significantly higher than that of normal pregnant mice (6.74%). Our results also showed that the number of cells expressing both CD25 and Foxp3 in URSA group (group 2) was obviously lower than that in normal control group (group 1). Our findings suggest that low frequency of $CD4^+CD25^+Foxp3^+$ Treg in spleen may correlate with adverse pregnancy outcome.

In this study we examined the effects of adoptive transfer of $CD4^+CD25^-$ T-cells and $CD4^+CD25^+$ nTreg into miscarriage-prone mice. We found the percentage of embryos undergoing resorption was significantly decreased in the mice of group 7, whereas that decrease was not observed in group 6, indicating that expression of CD25 is critical for mitigating resorption. The number of splenic $CD4^+CD25^+Foxp3^+$ Treg in group 7 has increased significantly compared to group 6. Thus, the transfusion of nTreg can increase the number of Treg in abortion-prone mice and prevent the appearance of spontaneous abortion.

Treg can be divided into two main populations: thymus-derived naturally occurring nTreg and peripheral- derived endogenous iTreg or iTreg induced ex vivo from $CD4^+CD25^-$ T-cells. In humans and mice, $CD4^+CD25^+Foxp3^+$ Treg of the periphery and spleen have been considered as a mixed population comprising nTreg and iTreg, nTreg only constitute < 5% to 10% of $CD4^+$ T-cells (Guerin et al., 2009). Therefore, ex vivo induction of a novel class of Treg was thought to be useful techniques to gain sufficient numbers for potential therapy. Numerous researchers claim TGF-β has an ability to induce $CD4^+CD25^-$ T-cells to become $CD4^+CD25^+$ Treg in vitro. However Foxp3 expression, which is associated with the development and function of Treg (Guerin et al., 2009), is unstable (Schmidt et al., 2016). The lack of stable Foxp3 expression may contribute to unsustained normal pregnancy (Tritt et al., 2008).

The stability of Foxp3 was thought to be affected by methylation or acetylation. One school of thoughts is that gene methylation plays a role in the repression of Foxp3 expression in activated T-cells (Kalekar et al., 2016), as the upstream CpG- rich enhancer of the Foxp3 gene in TGF-β-induced Treg and $CD4^+CD25^-$ T-cells is methylated, whereas nTreg remains relatively demethylated (Lal and Bromberg, 2009; Lal et al., 2009). On the other hand, other groups have recently observed that the methylation status in Foxp3 gene loci does not affect Foxp3 stability in vitro and in vivo. Rather, the stability and maintenance of iTreg related to acetylation of Foxp3 histone. Foxp3 protein has a short half-life. Acetylated Foxp3 would not subject to proteasomal degradation and maintain Foxp3 levels (Lu et al., 2011; Licciardi and Karagiannis, 2012).

HDACs influence gene expression through dampening histone-DNA and non-histone protein interactions. HDAC inhibitor, TSA, inhibits class I, II, and IV HDAC families of enzymes, leading to a more open chromatin architecture and access for transcription factors (Mclaughlin and La Thangue, 2004; Sugimoto et al., 2014). It has been reported that TSA directly modulates the acetylation state of Foxp3 and enhances the effect of TSA on Treg generation from $CD4^+CD25^-$ T cells (van Loosdregt et al., 2010; Lu et al., 2010). Recent studies have also shown that TSA strengthens the suppressive activity of Treg, thus causing more potent induction of iTreg (Kwon et al., 2012).

In our study, we treated splenic $CD4^+CD25^-$ T-cells with TSA ex vivo. We observed that treatment with TSA resulted in a higher frequency of $CD25^+Foxp3^+$ T-cells in the $CD4^+$ T-cells of the miscarriage-prone mating. There was an increasing trend in output of $CD4^+CD25^+Foxp3^+$ T-cells in vitro as dose of TSA increased from 1 to 100 nmol/L. Dose of TSA higher than 100 nmol/L might led to reduction of iTreg. The expression of CD25 and Foxp3 of iTreg induced by TSA is similar to nTreg. We also found that TSA treatment up-regulated expression of PD-1 and CTLA-4, and increased secretion of TGF-β and IL-10. These factors may help to establish an environment in favor of the maintenance of pregnancy-tolerance, thereby mitigating spontaneous abortion. Taking together, these results indicated TSA treatment in vitro increased the population of $CD4^+CD25^+Foxp3^+$ T cells and enhanced the functions of iTreg.

Besides TSA treatment, signaling induced by IL-2 and TCR stimulation is important, as well, for the induction of Treg- associated markers (Foxp3, CTLA-4, PD-1, TGF-β and IL-10). Therefore, we used anti-CD3 mAb and anti-CD28 mAb to stimulate TCR activation and induced Treg differentiation in the presence of IL-2. The iTreg with TCR stimulation alone expressed higher levels of CD25, Foxp3, CTLA-4 and PD-1 than the $CD4^+CD25^-$ T-cells without TCR stimulation. The higher levels of TGF-β and IL-10 after TCR stimulation also increased Treg suppressive capability.

Importantly, in vivo we showed that transfer of those TSA- induced Treg significantly reduced resorption rate and increased fetus survival. It has been reported that transfer of Treg fails to prevent miscarriage if carried out on day 7 of pregnancy. Our findings have indicated that transfusion with iTreg in the implantation phase (day 4, group 4) and early stage (day 1, group 3) of pregnancy is effective. Moreover, the rates of fetal resorption in group 3 were significantly lower than that in group 4. Adoptive transfer of Treg at an early stage of pregnancy may stem from the continual proliferation of transferred iTreg and re-establishing immune tolerance in CBA/J mice. There is some evidence that TSA- induced Treg shows regulatory activity on allogeneic T lymphocytes (Moon et al., 2009).

Constitutive expression of Foxp3 in iTreg is required for stable suppressive function. To further confirm the effect of TSA on Foxp3 stability, we analyzed the level of splenic $CD4^+CD25^+Foxp3^+$ Treg freshly isolated from CBA/J mice on Day 9 or Day 13 after injection of iTreg. Our results revealed that the percentage of $CD4^+CD25^+Foxp3^+$ T-cells in $CD4^+$ T-cells from group 3 or group 4 was significantly higher than that in group 2 or group 5. There was no difference in the population of $CD25^+Foxp3^+$iTreg between groups 3 and 4. Those results suggested that TSA might stabilize Foxp3 of iTreg for more than 13 days in vivo, which was beneficial to maintaining normal pregnancy.

In summary of this study, we report that inhibition of HDACs by TSA can induce functional $CD4^+CD25^+Foxp3^+$ Treg. However, the molecular mechanism remains unclear. Our preliminary experiments demonstrated that adoptive transfer of iTreg with TSA treatment in vitro was critical for maintenance of the tolerant state during pregnancy. These findings have potential applications in cell-based immunotherapy of human URSA.

Authors' roles

All authors fulfill the criteria for authorship, J.W. and J.Y. collected and analyzed the data, drafted and corrected the manuscript; Y.Q.Y performed the flow cytometry analysis; Z.Y.Z performed the adoptive transfer experiment; Y.Q.M performed the ELISA tests; X.Y.W and L.H.L assisted the experiments and preparation of the manuscript; F.X.Z and J.H.Z conceived the study plan and supervised the project; Y.L.C helped with experimental design and data presentation, prepared and edited the manuscript. All authors commented on the draft, have seen and approved the final version.

Funding

The authors' research is supported by Scientific and Technological Innovation Programs of Higher Education Institutions in Shanxi, China (2017165), Basic Research Projects of Technology Bureau in Datong City (2016115) and National Natural Science Foundation of China (81170621).

Disclosures

The authors have no financial conflict of interest.

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