Immunoregulation of PD-1/PD-L1 Inhibitory Pathway on Fetomaternal Tolerance in abortion Triggered by thyroid autoimmunity

Haixia Liu

Second Affiliated Hospital of Dalian Medical University

Mengya Chen

The Second Hospital of Dalian Medical University

Zhongyan Shan (✉ shanzhongyan@medmail.com.cn)

<https://orcid.org/0000-0002-2849-2380>

Weiping Teng

The First Hospital of China Medical University

Research

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Abstract

Background: TAI in euthyroid pregnant women is associated with miscarriage. Studies have shown that the PD-1/PD-L1 signaling pathway plays an important role in maternal-fetal tolerance by promoting Treg cells development and inhibition of Th17 cells responses, whereby it is essential for normal pregnancy maintenance. However, whether the PD-1/PD-L1 pathway leads to a Treg/Th17 imbalance has not been fully investigated in TAI.

Methods: TAI fetal loss model was established by thyroglobulin (mTg) immunized CBA/J female mice. The frequencies of splenic Th17, Treg, PD-1 and PD-L1 were tested by flow cytometry. IL-17, Foxp3, ROR γ t, PD-1 and PD-L1 mRNA levels were tested by real-time PCR.

Results: Compared with the control group, the number of CD4 + CD25 + Foxp3 + T lymphocyte in the placenta and spleen were significantly reduced ($P < 0.05$) in the experimental group (mTg group), CD4 + IL-17 + T-cell subsets and expression of ROR γ t and IL-17 mRNA in the placenta were increased ($P < 0.05$), the ratio of Treg/IL-17 in the placenta and spleen was decreased ($P < 0.05$). The expression of PD-1 and PD-L1 in mice immunized with mTg in CD4 + T group decreased the subset of cells in the placenta and spleen including Tregs.

Conclusions: The role of PD-1/PD-L1 pathway in an isolated thyroglobulin antibodies (TgAb) positive mouse abortion model is that it may lead to a peripheral Treg/Th17 imbalance and the maternal-fetal tolerance balance breakdown, which may ultimately result in fetal loss.

Plain English Summary

TAI with euthyroid is associated with miscarriage. However, the exact mechanism remains unclear. Studies have shown that the programmed cell death-1 (PD-1)/PD-1 ligand (PD-L1) pathway maintains normal pregnancy by regulating the regulation of T(Treg) cell development and inhibiting Th17 cell responses. As a negative conciliatory signal, fetal-maternal tolerance establishment and maintenance of pregnancy are achieved by regulating the T cell homeostasis through the interaction between PD-1 and PD-L1. Established a TAI fetal loss model after murine thyroglobulin (mTg) immunized CBA/J female mice mating with Balb/c males. The frequencies of splenic Th17, Treg, PD-1 and PD-L1 were tested by flow cytometry. Interleukin-17 (IL-17), forkhead box P3 (Foxp3), orphan retinoic acid nuclear receptor (ROR γ t), PD-1 and PD-L1 mRNA levels were tested by real-time PCR. Our findings demonstrated that PD-L1 expression in Treg cells in abortion mice with thyroiditis was positively correlated with the population of Treg cells, which suggest that the observed down-regulation of Treg cells and their differentiation and development function may contribute to pregnancy loss through PD-1/PD-L1 pathway.

Introduction

The most important feature of TAI is the presence of thyroid antibodies, including TPO-Ab and TgAb, either in clinical or subclinical thyroid dysfunction cases [1]. AITD are recognized as a subtype of T cell

mediated autoimmune diseases wherein the key elements of pathogenesis are considered to be Treg and Th17 cells [2]. PD-1/PD-L1 inhibitory pathway, which can weaken the response of T cells and promote the inhibitory signal pathway of T cell tolerance in the B7-CD28 family, causes stronger and wider immune depression [3]. PD-1 (CD279) is widely expressed, and it has two ligands known to bind: PD-L1 and PD-L2. Many studies have indicated that PD-L2 blockade did not show the immunoregulation, while PD-L1 is constitutively expressed on a variety of cell types, and at sites of immune privilege including the placenta, which hints that PD-L1 plays a key role in maternal-fetal immune tolerance. More importantly, a significant proportion of normal full-term human placental CD4 cells are PD-1 positive, suggesting that the PD-1/PD-L1 signaling pathway plays a key role in maintaining normal pregnancy [4].

The pathogenesis of increased abortion rates in women with thyroid autoimmunity remains hypothetical. TAI may represent a sign of a widespread autoimmune imbalance that leads to an increased risk of miscarriage, but is not the actual cause of miscarriage [1, 5, 6].

As a negative conciliatory signal, fetal-maternal tolerance establishment and maintenance of pregnancy are achieved by regulating the T cells homeostasis through the interaction between PD-1 and PD-L1 [7]. PD-1/PD-L1 negative conciliatory pathway plays a pivotal role in promoting the development of Tregs, in enhancing the activity of Tregs in the inflammatory microenvironment, and in regulating the activity of effector T cells effectively [8].

It is well accepted that CD4⁺CD25⁺ regulatory T cells (CD4⁺CD25⁺Treg) is a subset of CD4⁺T that has an immune regulation function. Attenuation of the PD-1 / PD-L1 signaling pathway reduces CD4⁺CD25⁺Treg cells function and attenuates down-regulation of Th17 cells, thereby increasing the activity of Th17 cells that are harmful to early pregnancy embryos. Animal experiments show that inhibition of PD-1/PD-L1 pathway increases abortion rate. Blocking PD-L1 reduced the ratio of Treg cells and effector T cells in the spleen and lymph nodes of mice, while increasing Th17 and Th1 cytokine levels [9]. RM women with abnormal immune cells, administration of IVIG during pregnancy can enhance Treg cells development which in turn reduces Th17 cells response, thus affecting Th17/Treg ratio in peripheral blood [10]. The ratio of Treg cells was increased in the peripheral blood and decidua during normal pregnancy, while the proportion of Tregs in peripheral blood of women with RM was significantly lower than that in normal non-pregnant women; the proportion of IL-17 in deciduous tissue was higher than that of peripheral blood during normal pregnancy, which suggests that physiological level of cytokines secreted by Th17 is conducive to the maintenance of pregnancy, while the proportion of Th17 in decidua and peripheral blood of unexplained recurrent spontaneous abortion women increased significantly, indicating that it is associated with pregnancy loss [9]. All the above results suggest that the maintenance of immune tolerance during normal pregnancy is determined by the balance of maternal Treg/Th17 cells.

In the present study, we aimed to evaluate the effects of PD-1/PD-L1 inhibitory pathway on maternal-fetal tolerance in abortion triggered by thyroid autoimmunity.

Materials And Methods

Immunisation protocols

Two hundred SPF female CBA/J mice (aged 4 weeks) were purchased from experimental animal research institution of Peking Union Medical College, Chinese Academy of Medical Sciences (Beijing, China HuaFukang Biological Technology Co, Ltd. Marketing Department). All experiments were conducted in accordance with the CAMS Guide for the care and use of laboratory animals. mTg was prepared from frozen mouse thyroids (KM mouse), as described by Imaizumi et al [11]. In order to induce autoimmune thyroiditis, CBA/J mice were first immunised with mTg (75µg/mouse) in complete Freund's adjuvant at 5 weeks of age and were then challenged with mTg (75µg/mouse) in incomplete Freund's adjuvant at 7 weeks of age. The same dose of PBS instead of mTg was used to immunize the control group mice, and the other methods were the same as those of the mTg group. Thereafter, CBA/J females were mated with Balb/c males after the booster dose of immunization for four weeks, then the presence of the female vaginal mucus plug was used to determine the 0.5th day of pregnancy. The mice bled and died on day 13.5 of pregnancy.

Thyroid function tests

TT4 and TSH in mouse serum was measured by an electrochemiluminescence immunoassay method (Immulite 1000, DPC, USA). TSH of control mice and TT4 were measured individually. Functional sensitivity of the TT4 assay was 1µg/dL. The intra-assay CV of serum TSH and TT4 were 1.23-3.38% and 1.26-3.20% respectively. CV values were 1.57-4.93% and 3.58-6.67% respectively.

Measurement of TgAb production

TgAb was determined by Enzyme Linked Immunosorbent Assay (ELISA) (R&D, USA). 100µL of 10µg/mL mTg in coating buffer was incubated overnight at 4°C in a 96-well plate. After washing and blocking with 100ul 1% BSA, 100µL of the diluted sera from immunized mice was incubated for 30min at 37°C followed by incubation with horseradish peroxidase-labeled sheep antimouse IgG (Sigma; 1:2000 dilution) and developed with p-nitrophenol phosphate (Sigma). The absorbance was measured at 450nm, and the experiments were performed twice.

Flow cytometry to determine cell expression

Fresh mouse placenta and spleen were prepared as single nuclear cell suspensions with 5 ml of 5% heat-inactivated FCS -containing RPMI 1640 medium (5%; HyClone). Cells were stained with the corresponding antibodies (both purchased in BD, USA) and then flow cytometry was performed on a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA, USA) to examine the expression of Treg, Th17, PD-1, PD-L1 in fresh or cultured T cells.

Real-time PCR analysis of mRNA expression

Total RNA was extracted from purified spleen cells and placental cells, then was reverse transcribed to cDNA; qRT-PCR was performed on a 9700 Sequence Detection System (Applied Biosystems, ABI) by using

the SYBR (Synergy Brands) Green Master Mix (Applied Biosystems) as previously described [12]. In this experiment, GAPDH was used as a reference gene. The primer sequences of the target gene and the reference gene were as follows:

PD-1

F: 5'-TGGCAATCAGGGTGGCTTC-3'(forward),

R: 5'-GACTCAGGCGGTTCAGTTCA-3'(reverse).

PD-L1

F: 5' -AGCGAATCACGCTGAAAGTCAA-3'(forward),

R: 5'-GGATAACCCTCGGCCTGACATA-3'(reverse).

GAPDH

F: 5'- ACTCCACTCACGGCAAATTC-3'(forward),

R: 5'-TCTCCATGGTGGTGAAGACA-3'(reverse).

Each gene was set five gradient standards and negative controls.

Statistical processing

Statistical analysis was performed using the SPSS v16.0 software package. All experiments were performed more than 3 times to ensure their authenticity. Data was analyzed by Student's t-test or χ -square test. Results are reported as AOR with 95%CI. $P < 0.05$ was considered to be statistically significant.

Results

Fetal resorption rates and the levels of TSH, TT₄ and TgAb in serum

Fetal resorption rates were increased in mTg group compared to the control group (45.63% vs. 3.1%; $P < 0.05$ Fig1). TSH and TT4 levels in both control group and mTg group did not show significant differences ($P < 0.05$ Table 1). Serum TgAb ELISA test showed that TgAb of the mice in mTg group was enhanced and significantly higher than in the control group, $P < 0.05$ Table 2).

Flow cytometry to determine cell expression

Treg cells in the Placenta and Spleen

The proportion of Treg cells in the placenta and spleen was evaluated in mTg group and control group by flow cytometry. The results in Fig.2 showed that the percentage of Treg cells in the placenta in mTg group was lower than that of control group ($3.39 \pm 1.82\%$ vs. $6.70 \pm 1.79\%$; $P < 0.05$). No significant differences were found in the frequency of $CD4^+CD25^-Foxp3^+$ and $CD4^+CD25^-Foxp3^-$ as percentage of $CD4^+$ T cells between mTg group and control group ($91.23 \pm 2.45\%$ vs. $87.60 \pm 2.26\%$; $P > 0.05$) and $5.47 \pm 2.21\%$ vs. $5.71\% \pm 1.79\%$; $P > 0.05$). In accordance with the following results, we also found that the proportion of Treg cells in the spleen in mTg group was lower than that of control group ($2.91 \pm 0.76\%$ vs. $4.72 \pm 0.57\%$; $P < 0.05$). Meanwhile, no significant differences were found in the frequency of $CD4^+CD25^-Foxp3^+$ as a percentage of $CD4^+$ T cells between mTg group and con group ($87.12 \pm 3.34\%$ vs. $88.33 \pm 3.22\%$; $P > 0.05$). There was also no apparent difference in the frequency of $CD4^+CD25^-Foxp3^-$ as a percentage of $CD4^+$ T cells between mTg group and control group ($8.96 \pm 1.27\%$ vs. $6.94 \pm 1.19\%$; $P > 0.05$).

Th17 cells in placenta and spleen

The proportion of placental Th17 cells in $CD4^+$ subsets in the mTg group was higher than that of Control group ($2.27 \pm 0.07\%$ vs. $1.37 \pm 0.09\%$; $P = 0.031$) (Fig.3.A,C), whereas the comparison of splenic Th17 cells between mTg group and Control group did not show statistically significant increases ($2.09 \pm 0.07\%$ vs. $1.76 \pm 0.13\%$; $P > 0.05$) (Fig.3.B,D)

The ratio of Treg/Th17 in the placenta and spleen

As shown in Figures 3E and F, the ratio of placental Treg/IL-17 was reduced in mTg group compared to Control group ($1.49 \pm 0.31\%$ vs. $4.89 \pm 0.24\%$; $P < 0.05$). Moreover, the ratio of Treg/IL-17 in the spleen in mTg group was significantly lower than that of Control group ($1.39 \pm 0.29\%$ vs. $4.45 \pm 0.36\%$; $P < 0.05$).

PD-1 expression in $CD4^+$ T cell subsets in the placenta and spleen

The expression of PD-1 in placenta and spleen was investigated by flow cytometry. Our results revealed that PD-1 expression in mTg group was reduced in $CD4^+$ T cell subset in the placenta compared with the control ($6.51 \pm 1.56\%$ vs. $18.22 \pm 2.12\%$; $P < 0.05$) (Fig.4.A,C). PD-1 expression in mTg group was reduced in $CD4^+$ T cell subset in the spleen compared with the control ($8.52 \pm 1.36\%$; $P < 0.05$ vs. $20.22 \pm 2.98\%$) (Fig.4.B,D).

PD-1 expression in Treg cells in placenta and spleen

We determined the expression of PD-1 in $CD4^+CD25^+Foxp3^+$, $CD4^+CD25^-Foxp3^+$ and $CD4^+CD25^-Foxp3^-$ cell subsets in the placenta and spleen by flow cytometry. The results showed that PD-1 expression on the placental Treg cells' surface in mTg group was significantly lower than that of Control group ($3.45 \pm 0.96\%$ vs. $37.60 \pm 5.26\%$; $P < 0.01$) (Fig.4.E,G), it was similar result in the spleen ($2.45 \pm 1.16\%$ vs. $33.60 \pm 3.26\%$; $P < 0.01$) (Fig.4.F,H). In the placenta, the percentage of PD-1 in $CD4^+CD25^-Foxp3^+$ and $CD4^+CD25^-Foxp3^-$ in mTg group and Con group was ($6.71\% \pm 0.99\%$ vs. $4.57 \pm 0.81\%$; $P > 0.05$) and ($7.60 \pm 1.16\%$ vs.

5.23 ± 1.05%; P>0.05) respectively; it was similar results in the spleen (7.72 ± 1.44% vs. 5.17 ± 1.28%; P>0.05) and (6.60 ± 1.27% vs. 4.23 ± 1.15%; P>0.05)

PD-L1 expression in placenta and spleen in Treg cells

Our results revealed that placental PD-L1 expression in CD4⁺ T cell subsets in mTg group was significantly reduced compared to that of Control group (15.11 ± 3.22% vs. 31.15 ± 2.46%; P<0.05) (Fig.5.A,C), it was similar results in spleen 18.45 ± 2.92% vs. 36.15 ± 3.76%; P<0.05) (Fig.5.B,D).

PD-L1 expression in CD4⁺ T cell subset in placenta and spleen

The results clearly showed that PD-L1 expression in the placental Treg cell surface in mTg group was significantly lower than that of Control group (15.79 ± 4.56% vs. 41.15 ± 4.06%; P<0.01) (Fig.5.E,G). But there were no significant differences of PD-L1 expression in CD4⁺CD25⁻Foxp3⁺ cells and CD4⁺CD25⁻Foxp3⁻ cells in the placenta of mTg group and Con group (5.37 ± 2.17% vs. 7.91 ± 1.99% and 8.33 ± 1.65% vs. 10.10 ± 2.47%; P>0.05). PD-L1 expression on splenic Treg cell surface in mTg group was significantly decreased in comparison with control group (19.19 ± 4.10% vs. 43.67 ± 3.76%; P<0.01) (Fig.5.F,H). There were no significant differences of PD-L1 expression in CD4⁺CD25⁻Foxp3⁺ cells and CD4⁺CD25⁻Foxp3⁻ cells in spleen of mTg group and Control group (8.99 ± 1.01% vs. 9.13 ± 1.87% and 7.07 ± 3.92% vs. 7.70 ± 2.91%; P>0.05).

Real-time PCR analysis of mRNA expression

Real-time amplification curve and corresponding amplification curve of each primer

The amplification power curve is S-shaped, with obvious exponential expansion period and plateau period. The whole curve runs smoothly (Fig.6.A), showing that the amplification result is ideal. The melting curve was analyzed by real-time PCR, the melting temperature was uniform, and the shape of the peak was sharp (Fig.6.B). It was confirmed that each primer was the only amplicon in the corresponding amplification product.

The mRNA level of Treg specific transcription factors and representative cytokines

The expression level of Treg cell-specific transcription factor Foxp3 and representative cytokine TGF-β mRNA in placental cells of mTg group was significantly lower than that of Control group (0.59 ± 0.17 vs. 1.01 ± 0.21; P<0.05) (Fig.7.A) and (0.73 ± 0.12 vs. 1.91 ± 0.15; P<0.05) (Fig.7.B). Similarly, the results clearly show that the mRNA level of specific transcription factor Foxp3 and the mRNA level of TGF-β in the spleen were consistent with that of the placenta (0.57 ± 0.31 vs. 1.18 ± 0.11; P<0.05) (Fig.7.C) and (0.53 ± 0.21 vs. 1.42 ± 0.35; P<0.05) (Fig.7.D).

The mRNA level of specific transcription factor RORγt and IL-17A in placenta and spleen

The expression level of Th17 cell-specific transcription factor ROR γ t and representative cytokine IL-17A mRNA in placental cells of mTg group were significantly higher than that of Control group (0.99 ± 0.05 vs. 0.37 ± 0.06 ; $P < 0.05$) (Fig.7.E) and (1.07 ± 0.11 vs. 0.56 ± 0.07 ; $P < 0.05$) (Fig.7.F). The expression levels of Th17 cell-specific transcription factor ROR γ t and representative cytokine IL-17A mRNA in spleen cells of mTg group were higher than those in Control group, but there were no statistical difference (1.13 ± 0.06 vs. 0.81 ± 0.13 ; $P > 0.05$) (Fig.7.G) and (0.91 ± 0.07 vs. 0.72 ± 0.11 ; $P > 0.05$) (Fig.7.H)

The mRNA level of PD-1 and PD-L1 in placenta and spleen

The mRNA levels of PD-1 and PD-L1 in the placenta and spleen in the mTg group and the Control group were determined by real-time RT-PCR. The mRNA level of PD-1 and PD-L1 in placenta in mTg group were significantly lower than that in the Control group (0.63 ± 0.13 vs. 1.51 ± 0.29 ; $P < 0.05$) (Fig.7.I) and (1.55 ± 0.31 vs. 2.23 ± 0.19 ; $P < 0.05$) (Fig.7.J), it was similar results in spleen (0.59 ± 0.13 vs. 1.33 ± 0.31 ; $P < 0.05$) (Fig.7.K) and (1.09 ± 0.35 vs. 2.11 ± 0.12 ; $P < 0.05$) (Fig.7.L)

Discussion

In this study, we demonstrated that Treg cells decreased in both the placenta and the spleen in an isolated TgAb positive mouse abortion model whereas Th17 cells only increased in the placenta with no statistically significant difference in the spleen, suggesting that the presence of Treg cells at the periphery and the maternal-fetal interface is important for the maintenance of normal pregnancy. As the population of Treg cells decreased, an imbalance in peripheral immunity and maternal-fetal tolerance may occur, eventually leading to fetal loss induced by thyroid autoimmunity whereas Th17 cells involvement in miscarriages only occurs at the site of maternal-fetal interface.

The maternal immune system's tolerance to the fetus is regulated by various mechanisms involving different immune cells, including peripheral immune cells and maternal-fetal interface local immune cells [13]. The Treg cells bank is further expanded in mice and humans in early pregnancy and can be found to have a very important protective effect when the maternal tissue first contacts antigens associated with invading placental trophoblast cells [14]. However, when the amplification of regulatory T cells responding to paternal antigens in mice is not sufficient, they may induce miscarriage [15]. Furthermore, recent studies have indicated that Treg cells play a more important role in the local regulation of fetal-specific immune responses at the fetal-maternal interface, since fetal-specific regulatory T cells can be preferentially recruited from maternal peripheral blood to fetal-maternal interfaces [16]. Treg cells quickly recruited uterine draining lymph nodes and activated in synthesis and allogeneic mating on the first day after embryo implantation [17]. Our data also suggest that the number of CD4⁺CD25⁺Foxp3⁺ T lymphocyte in the placenta and spleen was significantly reduced in mTg group compared with the control ($P < 0.05$), while there were no significant differences for the number of CD4⁺CD25⁻Foxp3⁺ and CD4⁺CD25⁻Foxp3⁻ in the placenta between the two groups ($P > 0.05$). All these results support the concept that regulatory T cells play an important role in maintaining the maternal-fetal immune tolerance.

Th17 cells are a recently discovered effector CD4⁺T cell subset that enhances acute inflammatory responses, expressing the transcription factor ROR γ and the pro-inflammatory cytokine IL-17 [18]. The effects of pregnancy results on the Th17 cells are not consistent [19, 20]. Nakashima et al [21], reported that Th17 cells accumulated at the decidua in miscarriage cases, and the number of Th17 cells was positively correlated with the number of neutrophils at the decidua; however, the difference in the number of Th17 cells between normal pregnant group and miscarriage group did not appear statistically significant. Studies by Wang et al [22, 23] have shown a significant increase in Th17 cells in peripheral blood and decidua in recurrent spontaneous abortion cases. In addition, the expression of not only IL-17 but also the essential transcription factor, ROR γ t, there was a significant increase in peripheral blood and decidual tissue of unexplained RM subjects compared to normal pregnant subjects. Our results demonstrated that Th17 cells increased at the feto–maternal interface in mTg group, meanwhile, the expression of characteristic transcription factor ROR γ t and representative cytokines IL-17A was also increased; however, there were no statistical differences of the above data in the spleen, which suggests that Th17 cells are only involved in the occurrence of abortion induced by thyroid autoimmunity at the feto-maternal interface; as such, these findings support the paper by Antje Habicht et al [24]. These results above indicated that heterologous antigen reactive T cells pool only expanded in partial placenta of deficient mice, rather than systematically expanding in the peripheral lymphoid organs such as spleen. Moreover, Th17 cells recruiting T cells in the local tissue may eventually lead to the fetal rejection.

PD-1/PD-L1 signaling pathway maintains normal pregnancy by promoting Treg cell development and inhibiting Th17 cell responses [25]. Notably, the regulation of ROR γ t/Foxp3 balance by cytokines determines the antigen stimulated naive T cells differentiation into Tregs or Th17. Xu et al [26], showed that Treg cells can be converted into Th17 cells in special cytokine milieu; thus, Tregs are not only involved in the differentiation of Th17 cells, but also can be converted into Th17 cells. Consequently, the balance between the two types of cells also plays a pivotal role in maintaining of maternal-fetal tolerance during pregnancy. Wang et al [22], reported a negative correlation between the number of Th17 and Treg cells in the peripheral blood and decidua. The ratio of Th17/Treg in miscarriages was significantly higher than that of non-pregnancy and normal pregnancy [27]. Wu et al [28], demonstrated that immunotherapy can increase the ratio of Treg/Th17 and play an important role in maintaining pregnancy. Our results also indicated that the ratio of Treg/Th17 in murine abortion group induced by thyroid autoimmunity was lower than that of the control group, and the ratio of the levels of characteristic transcription factors and representative cytokines was also decreased, which suggest that an imbalance of Treg/Th17 axis was involved in miscarriage induced by thyroid autoimmunity.

PD-1/PD-L1 pathway can confer maternal tolerance in allogeneic pregnancy models [7]. The latest research by our research team shows that the expression of PD-1 and PD-L1 in spleen and placenta in abortion mice with thyroiditis were decreased. In the work of Guleria et al [29], PD-L1 contributes to maternal tolerance by attenuating the ability of neutrophils to expand. If the PD-L1 signaling pathway is blocked in pregnant mice, the rejection rate of allogeneic receptors will increase. Defects in the PD-L1 gene cause an increase in fetal absorption rate and a decrease in survival rate [30]. Given the increase in

the abortion rate along with increased infiltration of placental T cells, we would predict that the deciduous cells-expressed PD-L1 inhibit maternal T cells-expressed PD-1, which occur due to the stimulation by paternal-antigens and then infiltrate the placenta.

Our findings demonstrated that PD-L1 expression in Treg cells in abortion mice with thyroiditis was positively correlated with the population of Treg cells, which suggest that the observed down-regulation of Treg cells and their differentiation and development function may contribute to pregnancy loss through PD-1/PD-L1 pathway [28, 29, 31]. Studies have shown that PD-L1 exert negative regulation effect on their maternal-fetal immunity tolerance by regulating the function of Treg cells. Resemble, Aluvihare et al [32], also reported that the blocking effect of PD-L1 antibody was dependent on the presence of Tregs, once we eliminated the Tregs, the blocking effect of PD-L1 antibodies disappears immediately. Recent data in vitro provide evidence that when co-culture of PD-L1-Ig beads with naive CD4⁺ T cell in the presence of anti CD3 and TGF- β , they can induce naive CD4⁺ T cell conversion into CD4⁺Foxp3⁺Tregs, in addition, PD-L1-Ig can also enhance the expression of Foxp3. Francisco et al [33], found that the PD-1/PD-L1 pathway controls peripheral T-cell tolerance in several ways: inhibiting expansion of naive self-assertive T cells and/or their differentiation into effector T cells; inducing Teff differentiation into Treg; inhibiting Teff directly; and the inhibiting Teff by activating Treg or DC. Francesca et al [34], found that blocking PD-L1 inhibited apoptosis of Ag-specific T cells and induced apoptosis of Tregs, promoted transformation into Th17 cells, and further impaired maternal-fetal tolerance.

In summary, PD-1/PD-L1 pathway, Treg cells and Th17 cells play fundamentally immunomodulatory role in maintaining maternal-fetal tolerance together, PD-1/PD-L1 pathway dysfunction, imbalance of Treg cells and Th17 cells axis in abortion mice with thyroiditis lead to the destruction of peripheral immune tolerance and maternal-fetal tolerance, ultimately contributing to maternal rejection of the fetus. Therefore, interfering with PD-1/PD-L1 pathway may provide a new strategy for strengthening the function of reg cells, thus maintaining immune tolerance in thyroid autoimmunity. But there are still some limitations in our study. Firstly, the mouse immune induction model does not fully represent the human thyroiditis pregnancy process, so our experimental group also hopes that in future experiments, Treg and Th17 cells can be sorted from the placenta of AIT pregnant patients for subsequent enhancement/inhibition experiments. Secondly, our study only focused on confirming the changes in Treg cells function through the expression of cytokines and the levels of transcription factors associated with Treg activity. No attempt was made to enhance the PD1-L1 pathway to observe changes in the number of Treg cells. In future experiments, we will delve into the relationship between PD-1/PD-L1 signaling pathway and Treg cells from various aspects.

Conclusion

The role of PD-1/PDL1 pathway in thyroid autoimmunity mouse abortion model may consist not only in controlling Treg cells development and function, but also in regulating the balance of Treg/Th17 cells, as well as the fetomaternal and peripheral immune tolerance balance breakdown, ultimately leading to the fetal loss.

Abbreviations

TAI: Thyroid autoimmunity, PD-1: Programmed cell death-1, PD-L1: Programmed cell death -ligand 1, Treg: regulatory T cells, Th17: T helper 17 cells, IL-17: Interleukin-17, Foxp3: Forkhead box P3, RORyt: orphan retinoic acid nuclear receptor, TgAb: Thyroglobulin antibodies, TPO-Ab: Anti-thyroid peroxidase, AITD: Autoimmune thyroid diseases, SPF: Specific Pathogen free, PBS: Phosphate buffered saline, TT4: Total thyroxine, TSH: Thyroid stimulating hormone, CV: coefficients of variation, FCS: fetal calf serum, RPMI: Roswell Park Memorial Institute, AOR: adjusted odds ratios, RM: recurrent miscarriage, IVIG: intravenous immune globulin, CAMS: Chinese Academy of Medical Sciences.

Declarations

Acknowledgments

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Ethics Statement

Animal protocols were approved by the Experimental Animal Ethics Committee of Dalian Medical University (approval number: L20160242), and all of the animals were treated in accordance with the Institutional Guidelines for Experiments Using Animals.

Author Contributions

Haixia Liu conceived and wrote the paper. Mengya Chen drafted the English manuscript and revised the diagrams in the article. Zhongyan Shan revised and approved the final manuscript. WeipingTeng evaluated and reviewed manuscript structure, ideas, and science.

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Availability of data and materials

All data generated or analysed during this study are included in this published article.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Contributor Information

Zhongyan Shan, shanzhongyan@medmail.com.cn

Weiping Teng, twp@vip.163.com

Haixia Liu, dllhx1017@163.com

Mengya Chen, cmy930617@163.com

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Tables

Table 1. Comparison of serum TSH and TT4 levels in each group (± S.E.M)

Groups	TSH[mIU/L]	TT ₄ [μg/dL]
mTg Group[n = 15]	0.22 ± 0.03	4.70 ± 0.91
Con Group[n = 15]	0.33 ± 0.05	5.11 ± 0.25

Table 2. Serum TgAb OD values of each group

OD	Con Group	mTg Group
Value	(n = 7)	(n = 7)
	0.032	1.123
	0.022	1.231
	0.025	1.098
	0.029	0.989
	0.056	1.213
	0.034	1.293
	0.036	1.089
Mean	0.034	1.148
Standard deviation	0.011	0.103

Figures

Figure 1

Comparison of fetal resorption rates in each group ($p < 0.05$)

Figure 2

Placental mononuclear cells (A); Splenic mononuclear cells (B); Representative flow cytometry analysis graph of CD4⁺ T cell subsets (CD25-Foxp3⁺, CD25-Foxp3⁻, CD25⁺Foxp3⁺) in mTg group and Con group in placental mononuclear cell (C) splenic mononuclear cell (D); The percentages of CD4⁺ T cell subsets in mTg group and Con group in placenta (E) and spleen (F);

Figure 3

The percentages of Th17 cells in CD4+ T cells in placenta (A) and spleen (B); Flow cytometry analysis of the percentage of Th17 cells in mTg group and con group in CD4+ subsets in placenta (C) and spleen (D). Imbalance of Treg/Th17 cells in the placenta and spleen in mTg group and con group. The percentages of Treg cells and Th17 cells in placenta (E) and spleen (F).

Figure 4

PD-1 expression in CD4+ T cells in the placenta and spleen. The percentage of PD-1 in mTg group and Con group in CD4+ T cells in placenta (A) and spleen (B); Flow cytometry analysis of PD-1 expression in mTg group and Con group in placenta (C) and spleen (D). PD-1 expression in the placental CD4+CD25-Foxp3+, CD4+CD25-Foxp3-, CD4+CD25-Foxp3- cell subsets in placental (E) and splenic (F)

CD4+ T cells. The expression of PD-1 in CD4+CD25+Foxp3+, CD4+CD25-Foxp3+, CD4+CD25-Foxp3- cell subsets in placenta (G) and spleen (H) was analyzed by flow cytometry.

Figure 5

The percentage of PD-L1 in mTg group and Con group in CD4+ T cells in placenta (A) and spleen (B). Flow cytometry analysis for the expression of PD-L1 in CD4+ T cell subsets in placenta (C) and spleen (D). PD-L1 expression in the placental CD4+CD25+Foxp3+, CD4+CD25-Foxp3+, CD4+CD25-Foxp3- cell

subsets in placental (E) and splenic (F) CD4+ T cells. The expression of PD-1 in CD4+CD25+Foxp3+, CD4+CD25-Foxp3+, CD4+CD25-Foxp3- cell subsets in placenta (G) and spleen (H) was analyzed by flow cytometry.

Figure 6

(A) Real-time amplification curve. (B) Identification of amplification products

Figure 7

mRNA level of Foxp3 (A), TGF- β (B), ROR γ t (E), IL-17A (F), PD-1(I) and PD-L1(J) in placenta in mTg group and Con group was determined by real-time RT-PCR. The mRNA level of Foxp3 (C), TGF- β (D), ROR γ t (G), IL-17A (H), PD-1(K) and PD-L1(L) in spleen in mTg group and Con group is determined by real-time RT-PCR.