The Fluctuation of Regulatory T Cells during Pregnancy and Obstetrical Complications

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Research

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Abstract

Background: Regulatory T cells (Tregs) are critical immunomodulators during pregnancy by preventing maternal T-cell activation against fetal cells. However, how characteristics of maternal Tregs vary during pregnancy is still unclear. We analyzed the proportion and phenotypic characteristics of peripheral blood Tregs in normal pregnant women, women with recurrent pregnancy loss (RPL) or gestational diabetes mellitus (GDM), and non-pregnant women.

Methods: We investigated the proportion of $CD4^+$ Tregs, $CD8^+$ Tregs and the expression of PD-1, GITR, HLA-G and CTLA-4 on them in the peripheral blood of normal pregnancies during 1st (n = 28), 2nd (n = 43), and 3rd trimester (n = 33); In addition, we evaluated pregnancies in the 1st trimester complicated by RPL (n = 21), in the 2nd (n = 17) and 3rd trimester (n = 28) complicated by GDM. Non-pregnant women (n = 57) were also investigated using flow cytometry.

Results: During normal pregnancy, the proportion of $CD4^+$ Tregs in all trimester and $CD8^+$ Tregs in 2nd and 3rd trimester were higher(P < 0.05respectively) compared with non-pregnancy women. Moreover, the proportion of $CD4^+$ Tregs was higher in 2nd trimester compared to 1st and 3rd trimester (P < 0.01) while the proportion of $CD8^+$ Tregs was higher in 3rd trimester compared to 1st and 2nd trimester (P < 0.05). Compared to non-pregnant studies, the proportion of $GITR^+/CD8^+$ Tregs and $HLA\text{-}G^+/CD8^+$ Tregs in all trimester were higher(P < 0.05, respectively). Moreover, the proportion of $PD\text{-}1^+/CD4^+$ Tregs, $GITR^+/CD4^+$ Tregs, $PD\text{-}1^+/CD8^+$ Tregs and $CTLA\text{-}4^+/CD8^+$ Tregs in 3rd trimester were significantly higher compared to 1st, 2nd trimester and non-pregnant group(P < 0.05, respectively).

In RPL and GDM groups, the proportions of $CD4^+$ Tregs in all trimesters were decreased while the proportions of $CD8^+$ Tregs in all trimesters were increased compared to normal pregnant group (P < 0.05 respectively).In RPL group, the proportion of $PD\text{-}1^+/CD4^+$ Tregs, $GITR^+/CD4^+$ Tregs and $HLA\text{-}G^+/CD4^+$ Tregs were decreased compared to 1st trimester normal pregnant group (P < 0.05respectively). In 2nd trimester GDM group, the proportion of $HLA\text{-}G^+/CD4^+$ Tregs were decreased compared to 2nd trimester normal pregnant group (P < 0.05respectively). In 3rd trimester GDM group, the proportion of $PD\text{-}1^+/CD4^+$ Tregs, $GITR^+/CD4^+$ Tregs, $PD\text{-}1^+/CD8^+$ Tregs, $GITR^+/CD8^+$ Tregs and $HLA\text{-}G^+/CD8^+$Tregs were decreased compared to 3rd trimester normal pregnant group (P < 0.05, respectively).

Conclusions: The proportion of $CD4^+$ Tregs and $CD8^+$ Tregs increased during pregnancy, the proportions and subsets of $CD4^+$ Tregs decreased and those of $CD8^+$ Tregs increased in pregnancies complicated by RPL and GDM, indicating that regulatory T cells play a role in pregnancy maintenance, and the abnormal expression of Tregs might be related to the complicated pregnancy.

Background

Regulatory T cells (Tregs) have been reported to be the important players in the immune tolerance of the fetus during pregnancy [1]. It has two main cell subtypes, $CD4^+$ Tregs and $CD8^+$ Tregs. $CD4^+$ Tregs play a dominant role in the maintenance of immunological self-tolerance by preventing immune and autoimmune responses against self-antigens [2]. The proportion of $CD4^+$ Tregs increased in early pregnancy, peaked in mid-pregnancy, and decreased in late pregnancy [3]. Reduced population of $CD4^+$ Tregs has been reported in women with recurrent pregnancy loss (RPL) or gestational diabetes mellitus (GDM) [4–9]. $CD8^+$ Tregs have regulatory functions in cancer, autoimmune diseases, and infectious diseases [10–12]. $CD8^+$ Tregs preferentially target activated T cells and exert its function in these ways: (i) direct killing of the target cell, (ii) negative signaling directly on the target or on APCs and (iii) secretion of soluble factors, such as immunosuppressive cytokines [13]. However, the characteristic of $CD8^+$ Tregs during pregnancy have not been studied well.

There are surface markers which might contribute to the function of $CD4^+$ and $CD8^+$ Tregs and participate in the immune regulatory mechanism of $CD4^+$ and $CD8^+$ Tregs, such as programmed cell death receptor-1 (PD-1/CD279), glucocorticoid-inducible TNF receptor family-related protein (GITR/CD357), human leucocyte antigen-G (HLA-G) and cytotoxic T lymphocyte antigen 4 (CTLA-4/CD152)[14].

PD-1 is a cell surface receptor belonging to the CD28 family. The PD-1/PD-L1 pathway may promote the development and function of $CD4^+$ Tregs, and inhibit the activation of effector T cells (Teff), thus regulate the T-cell homeostasis and the peripheral tolerance [15]. The use of anti-PD-1 antibodies would inhibit immune responses by both promoting $CD4^+$ Treg activity and inhibiting Teff responses [16]. Furthermore, the Th17/Treg immune balance in the feto-maternal interface is intimately associated with the PD-1/PD-L1 pathway [17]. Additionally, $PD\text{-}1^+/CD8^+$ Tregs generated in a cardiac allograft model are localized to graft, produce both IFN-γ and IL-4, and inhibit CD4 effector responses [18].

GITR expression can be observed at a high level in human and murine $CD4^+$ and $CD8^+$ Tregs following the activ

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ation [19–21]. GITR and its ligation can block suppression of $CD4^+$ Tregs and may increase $CD4^+$ Treg proliferation and enhance the survival of Teff [22, 23]. It has been reported that GITR acts as a costimulatory molecule for the activation of $CD4^+$ Tregs [24]. In $CD8^+$ T cells, the costimulatory role of

GITR is indispensable since its absence impairs the proliferation response of these cells to CD28 costimulation [25].

HLA-G is a nonclassical HLA class I molecule and immune tolerizing molecule. In allogeneic situations such as pregnancy, the expression of HLA-G has been related to a better acceptance of the fetus or the allograft [26], indicating that HLA-G^+ Tregs might be a distinct and important regulatory subset which is prominent in the context of pregnancy. HLA-G expressing $CD4^+$ Tregs demonstrated effective suppression of T-cell proliferation [27, 28]. There were results that showed an increase in the percentage of HLA-$G^+/CD8^+$ Tregs in HIV patients [29, 30], suggesting that these cells might be beneficial for immune homeostasis and the dysfunction of HLA-G^+ Tregs might be associated with immune dysregulation.

CTLA-4 can compete with CD28 for B7 binding and is known to be an important negative regulator of T-cell function. Both $CD4^+$ and $CD8^+$ Tregs rapidly express high levels of surface CTLA-4 following activation [31]. It directly delivers inhibitory signals through the cell-cell interaction that may support a successful pregnancy outcome by enhancing $CD4^+$ Tregs [32]. Moreover, the suppression of CTLA-$4^+/CD8^+$ Tregs could be relieved by blockade of CTLA-4 [31, 33].

The aim of this study was to investigate the proportion and phenotypic characteristics of $CD4^+$ Tregs and $CD8^+$ Tregs associated in different situations, to determine the role of Tregs during pregnancy and obstetrical complications.

Methods

Study population

The study was approved by the ethics committee of Yuhuangding hospital, and all study subjects signed informed consent prior to entering the study.

Totally 227 women were included in this study at Yuhuangding hospital from May 1, 2019 to October 1, 2019, including normal pregnancies during 1st (n = 28), 2nd (n = 43), and 3rd trimester (n = 33); pregnancies in the 1st trimester complicated by RPL (n = 21), in the 2nd (n = 17) and 3rd trimester (n = 28) complicated by GDM; and non-pregnant women (n = 57).

Peripheral blood was collected in 1st (normal pregnant women and women complicated by RPL at 7 - 9 weeks gestation), 2nd (normal pregnant women and women complicated by GDM at 24 - 28 weeks gestation), and 3rd trimester (normal pregnant women and women complicated by GDM at 37 - 41 weeks gestation) as well as non-pregnant fertile women.

RPL was defined as a couple having two or more consecutive pregnancy losses before week 24 of gestation without a history of normal pregnancy [34]. The diagnosis of GDM was confirmed between 24 and 28 weeks of gestation by a positive 2 - h 75 g oral glucose tolerance test with the following criteria: a

fasting plasma glucose ≥ 5.1 mmol/l (92 mg/dl), or a 1-h plasma glucose level of ≥ 10.0 mmol/l (180 mg/dl), or a 2-h plasma glucose of ≥ 8.5 mmol/l (153 mg/dl) [35].

Cells preparation and flow cytometry analysis

The following monoclonal antibodies (mAbs) were used to analyze Tregs: peridinin chlorophyll protein(PerCP)/Cy5.5-conjugated anti-CD3, fluorescein isothiocyanate FITC-anti-CD4, PE-conjugated anti-CD25, brilliant violet 510 anti-CD127, brilliant violet421 anti-CD152 (CTLA-4), allophycocyanin APC anti-HLA-G, APC/Fire750 anti-CD279 (PD-1), and PE/Cy7 anti-CD357 (GITR). For surface staining, cells were incubated with the respective mAbs for 15 min at room temperature in the dark according to the manufacturer's instructions (eBioscience, San Diego, C.A, USA). After that, cells were washed twice and suspended in PBS before analysis.

Cells were analyzed on a FACS Canto II flow cytometer (BD Biosciences, USA); 200,000 events were recorded. The gating strategy for the detection of cells was as follows: The population of $CD4^+$ Tregs was characterized by the gating of $CD3^+$, $CD4^+$, $CD25^+$, and $CD127^{low/-}$ subsets. The population of $CD8^+$ Tregs was characterized by the gating of $CD3^+$, $CD8^+$, $CD25^+$ and $CD127^{low/-}$ subsets. Then, within these subsets, GITR, PD-1, HLA-G or CTLA-4 cells were gated, respectively. The collected data were exported to flowJo software for analysis (Treestar, Ashland, OR).

Statistical analysis

Statistical analyses were performed using statistical software GraphPad Prism, version 5 (GraphPad, San Diego, CA, USA). Categorical data were presented as number and percentage using chi-square (X2) test to analyze them. Data were described as mean ± standard error (SEM). Normality tests were used first to determine if all the data sets are well-modeled by a normal distribution and to compute how likely it is for a random variable underlying the data set to be normally distributed. The differences between the two groups were analyzed by T-test. The differences between multiple groups were analyzed by one-way ANOVA. Statistical significance was set at P 0.05.

Results

Baseline characteristics

Our study included 104 normal pregnant (NP) women (including 28 first trimester, 43 second trimester, and 33 third trimester), 21 first trimester RPL, 45 GDM (including 17 second trimester GDM, 28 third trimester GDM) and 57 non-pregnant women. The demographic and clinical data for all the participants and their newborns are presented in Table 1.

Proportion of $CD3^+$, $CD4^+$, and $CD8^+$ T cells

During normal pregnancy, the proportion of $CD3^+$ T cells in 3rd trimester (76.26 ± 1.19) was significantly higher compared to 1st, 2nd trimester and non-pregnant group (64.56 ± 1.45, 69.27 ± 1.88, and 67.83 ±

1.55) (P 0.01, respectively). Moreover, the proportion of $CD3^+$ T cells in 2nd trimester (70.55 ± 1.23) was higher compared to 1st trimester (64.56 ± 1.45) (P 0.01). In 3rd trimester GDM group, the proportion of $CD3^+$ T cells (70.31 ± 1.25) was decreased compared to 3rd trimester normal pregnant group (76.26 ± 1.19) (P 0.01) (Fig. 1A). There was no significant difference in the proportion of $CD3^+$ T cells in RPL group compared to other groups and those of $CD4^+$ and $CD8^+$ T cells in different groups (P 0.05, respectively). (Fig. 1B C)

Proportion of $CD4^+$ Tregs and $CD8^+$ Tregs

During normal pregnancy, the proportions of $CD4^+$ Tregs in all trimesters (6.38 ± 0.39, 7.67 ± 0.14, and 6.38 ± 0.51) were significantly higher compared to the non-pregnant group (5.06 ± 0.23) (P 0.05, respectively). The proportion of $CD4^+$ Tregs in 2nd trimester (7.67 ± 0.14) was significantly higher compared to 1st and 3rd trimester (6.38 ± 0.39, 6.38 ± 0.51) (P 0.05, respectively) (Fig. 2A,C). The proportions of $CD4^+$ Tregs in 1st trimester RPL group, 2nd and 3rd trimester GDM group (5.26 ± 0.33, 4.87 ± 0.36, and 4.25 ± 0.39) were significantly decreased compared to the same trimester of normal pregnant group respectively (6.38 ± 0.39, 7.67 ± 0.14, and 6.38 ± 0.51) (P 0.05, respectively). (Fig. 2C).

During normal pregnancy, the proportion of $CD8^+$ Tregs was gradually higher. Compared to non-pregnant group (0.21 ± 0.02), the proportion of $CD8^+$ Tregs in 2nd (0.61 ± 0.06) and 3rd trimester pregnant group (0.74 ± 0.13) were significantly higher (P 0.01, respectively), however, there was no significant difference in 1st trimester pregnant group (0.32 ± 0.05) (P0.05). Moreover, the proportion of $CD8^+$ Tregs in 2nd (0.61 ± 0.06) and 3rd trimester pregnant group (0.74 ± 0.13) were significantly higher compared to 1st trimester pregnant group (0.32 ± 0.05) (P 0.05, respectively) (Fig. 2B,D). The proportion of $CD8^+$ Tregs in 1st trimester RPL group, 2nd and 3rd trimester GDM group (0.62 ± 0.08, 1.27 ± 0.21, and 1.09 ± 0.19) were significantly higher compared to the same trimester of normal pregnant group respectively (0.32 ± 0.05, 0.61 ± 0.06, and 0.74 ± 0.13) (P 0.05, respectively) (Fig. 2D).

Expression of PD-1, GITR, HLA-G and CTLA-4 on $CD4^+$ T cells

There was no significant difference in the proportion of PD-$1^+/CD4^+$ T cells in the different trimester of normal pregnant group(P 0.05, respectively).Moreover, the proportion of PD-$1^+/CD4^+$ T cells in RPL and GDM group had no significant difference compared with normal pregnant group (P 0.05, respectively).

The proportion of GITR $^+/CD4^+$ T cells increased after pregnancy (P 0.01, respectively). During normal pregnancy, the proportion of $GITR^+/CD4^+$ T cells in 3rd trimester (1.95 ± 0.30) was significantly higher compared to 1st (0.68 ± 0.10) and 2nd trimester (0.88 ± 0.14) (P 0.01, respectively). The proportion of $GITR^+/CD4^+$ T cells in 1st trimester RPL group, 2nd and 3rd trimester GDM group (0.41 ± 0.07, 0.41 ± 0.09, and 0.91 ± 0.26) were decreased compared to the same trimester of normal pregnant group (0.68 ± 0.10, 0.88 ± 0.14, and 1.95 ± 0.30) (P 0.05, respectively).

Compared to the non-pregnant group, there was no significant difference in the proportion of HLA-G$^+$/CD4$^+$ T cells in normal pregnant group (P 0.05, respectively). Moreover, there was no significant difference in the proportion of HLA-G $^+$/CD4$^+$ T cells in the different trimester of normal pregnant group (P 0.05, respectively). The proportion of HLA-G$^+$/CD4$^+$ T cells in 1st trimester RPL group, 2nd and 3rd trimester GDM group (0.28 ± 0.03, 0.26 ± 0.06, and 0.21 ± 0.14) were decreased compared to the same trimester of normal pregnant group (0.45 ± 0.06,0.52 ± 0.05, and 0.76 ± 0.11) (P 0.05, respectively).

There was no significant difference in the proportion of CTLA-4 $^+$/CD4$^+$ T cells in different groups (P 0.05, respectively) (Table 2).

Similar results were found in CD3$^+$ and CD8$^+$ T cells (supplemental table 1, 2).

Expression of PD-1, GITR, HLA-G and CTLA-4 on CD4$^+$ Tregs

The proportion of PD-1$^+$/CD4$^+$ Tregs and GITR$^+$/CD4$^+$ Tregs in 3rd trimester normal pregnant group (6.29 ± 0.81, 6.66 ± 0.75) were higher compared with non-pregnant group (2.87 ± 0.33, 4.24 ± 0.31), 1st and 2nd trimester normal pregnant group (P 0.01, respectively). The proportion of PD-1$^+$/CD4$^+$ Tregs and GITR$^+$/CD4$^+$ Tregs in 1st trimester RPL group and 3rd trimester GDM group were decreased compared to the same trimester of normal pregnant group (P 0.05, respectively). There was no significant difference in the proportion of PD-1$^+$/CD4$^+$ Tregs in 2nd trimester GDM group compared with 2nd trimester normal pregnant group (P 0.05, respectively).

Compared to non-pregnant group, there was no significant difference in the proportion of HLA-G $^+$/CD4$^+$ Tregs in normal pregnant group (P 0.05, respectively). Moreover, there was no significant difference in the proportion of HLA-G$^+$/CD4$^+$ Tregs in the different trimester of normal pregnant group (P 0.05, respectively). The proportion of HLA-G$^+$/CD4$^+$ Tregs in 1st trimester RPL group and 2nd trimester GDM group (1.65 ± 0.28, 1.54 ± 0.28) were decreased compared to the same trimester of normal pregnant group (2.84 ± 0.39, 3.71 ± 0.56) (P 0.05, respectively). There was no significant difference in the proportion of HLA-G $^+$/CD4$^+$ Tregs in 3rd trimester GDM group compared with 3rd trimester normal pregnant group (P 0.05).

There was no significant difference in the proportion of CTLA-4$^+$/CD4$^+$ Tregs in different groups (P 0.05, respectively)(Table 3).

Expression of PD-1, GITR, HLA-G and CTLA-4 on CD8$^+$ Tregs

The proportion of PD-1$^+$/CD8$^+$ Tregs in 3rd trimester (18.74 ± 2.61) were significantly higher compared to non-pregnant group (5.35 ± 0.17), 1st and 2nd trimester normal pregnant group (10.43 ± 2.86, 5.63 ± 1.13) (P 0.01, respectively) (Table 4). The proportion of PD-1$^+$/CD8$^+$ Tregs in 3rd trimester GDM group (8.49 ± 2.45) was decreased compared to 3rd trimester normal pregnant group (18.74 ± 2.61) (P 0.05).

There were no significant difference in the proportion of PD-1$^+$/CD8$^+$ Tregs in 1st trimester RPL and 2nd trimester GDM group compared to the same trimester of normal pregnant group (P ▯ 0.05, respectively).

The proportion of GITR$^+$/CD8$^+$ Tregs increased after pregnancy (P ▯ 0.01, respectively). The proportion of GITR$^+$/CD8$^+$ Tregs in 3rd trimester (25.25 ± 3.21) was significantly higher compared to 1st and 2nd trimester normal pregnant group (14.92 ± 3.23, 10.94 ± 1.79) (P ▯ 0.01, respectively). The proportion of GITR$^+$/CD8$^+$ Tregs in 3rd trimester GDM group (8.58 ± 2.56) was decreased compared to 3rd trimester normal pregnant group (25.25 ± 3.21) (P ▯ 0.05). There was no significant difference in the proportion of GITR $^+$/CD8$^+$ Tregs in 1st trimester RPL or 2nd trimester GDM group compared with the same trimester of normal pregnant group (P ▯ 0.05, respectively).

The proportion of HLA-G$^+$/CD8$^+$ Tregs increased after pregnancy (P ▯ 0.01, respectively), but it has no obvious fluctuation during pregnancy (P ▯ 0.05, respectively).The proportions of HLA-G $^+$/CD8$^+$ Tregs in 2nd and 3rd trimester GDM group (5.27 ± 1.81, 9.01 ± 3.09) were decreased compared to the same trimester of normal pregnant group (12.38 ± 1.88, 16.87 ± 2.13) (P ▯ 0.05, respectively). There was no significant difference in the proportion of HLA-G $^+$/CD8$^+$ Tregs in 1st trimester RPL group compared with 1st trimester normal pregnant group (P ▯ 0.05).

The proportion of CTLA-4$^+$/CD8$^+$ Tregs in 3rd trimester normal pregnant group (6.82 ± 1.62) was higher compared with non-pregnant group (1.65 ± 0.67) (P ▯ 0.01). There was no significant difference in the proportion of CTLA-4$^+$/CD8$^+$ Tregs in the different trimester of normal pregnant group (P▯0.05, respectively). There was no significant difference in the proportion of CTLA-4$^+$/CD8$^+$ Tregs in RPL and GDM group compared with the same trimester of normal pregnant group (P ▯ 0.05, respectively) (Table 4).

Discussion

During pregnancy, the maternal immune system changes in order to maintain immune tolerance towards the paternal antigen expressed on fetal cells. T lymphocytes, which play an important role in the prevention of external pathogen infection and the maintenance of environmental stability, have major regulatory functions in immune responses [36]. Our experimental results showed that during pregnancy, the proportion of CD3$^+$ T cells was low in 1st trimester, increased in 2nd trimester, and reached the highest in 3rd trimester. In 3rd trimester GDM group, the proportion of CD3$^+$ T cells was lower than that in normal pregnant group. However, the proportion of CD4$^+$ and CD8$^+$ T cells in normal pregnant group and GDM group showed no significant difference, which is in line with previous studies [37-39].

Tregs play an important role in maternal-fetal immune tolerance. In this article, we mainly analyzed CD4$^+$ Tregs and CD8$^+$ Tregs. Previously, we reported that the proportions of CD4$^+$ Tregs in peripheral blood (PB) and decidua were decreased in women with unexplained RPL[4]. The temporary elevation of CD4$^+$ Tregs on the day of embryo transfer was associated with the higher embryo implantation rate [40]. Adoptive transfer of CD4$^+$ Tregs reverses the increase of abortion rate in abortion-prone mice model [41]. In this

study, we found that the proportion of $CD4^+$ Tregs was higher during pregnancy compared with that in non-pregnant women. Moreover, there is an increase in circulating $CD4^+$ Tregs during 1st trimester, peaking during 2nd trimester and then a decrease during 3rd trimester, the proportion of $CD4^+$ Tregs were significantly lower in cases of RPL and GDM than that in normal pregnancy, which is consistent with previous studies [3].

$CD8^+$ Tregs exert immune-regulatory function and maintain the homeostasis and establishment of immune function. $CD8^+$ T cells are the main component of decidual T cells and $CD8^+$ Tregs, which accumulate in decidua during early pregnancy, and have been evidenced to be important in the maintenance of a normal pregnancy [42, 43]. In the present study, $CD8^+$ Tregs in normal pregnancy increased during 1st trimester, but there was no significant difference between 1st trimester and non-pregnant group. Then it increased significantly during 2nd trimester and peaked during 3rd trimester. These data showed that $CD8^+$ Tregs might exert maximum suppressive function during middle and late pregnancy which might be due to the continuous exposure of antigens of the developing fetus. It has been reported that the proportion of $CD8^+$ Tregs in the decidua of normal pregnant women was significantly higher than that in peripheral blood [42]. Women with preeclampsia (PE) exhibited lower level of $CD8^+$ Tregs in PB at about 32 weeks of pregnancy compared to normal pregnant control[44]. In our study, we found that the proportion of $CD8^+$ Tregs in women with RPL or GDM was significantly higher compared to 1st and 2nd trimester normal pregnant women.

Tregs exert their immunosuppressive activities through the secretion of anti-inflammatory cytokines, such as TGF-β and IL-10, cell-to-cell interaction, or the expression of other markers, such as PD-1, GITR, HLA-G and CTLA-4. Although these Tregs markers are not specific, they are essential tools for defining subset of Tregs and associated with Tregs functionality. In the present study, we investigated whether Treg subsets varied in the peripheral blood of normal pregnant women and women with RPL or GDM.

PD-1 has been described to be important for both $CD4^+$ and $CD8^+$ Tregs function [45-48]. Additionally, $CD4^+$ Tregs and $CD8^+$ Tregs suppress $CD4^+$ Teff responses directly through PD-1/ PD-L1 axis [49-51]. In a heart transplantation model, adding $PD\text{-}1^+$/ $CD8^+$ Tregs to the graft can produce interferon-γ (IFN-γ) and interleukin-4 (IL-4) to inhibit the $CD4^+$ T cells response [18]. In women with RPL or GDM, the expression of PD-1 has been reported to be decreased [52, 53].

In the present study, we found that in normal pregnant women, the proportions of $PD\text{-}1^+/CD4^+$ Tregs and $PD\text{-}1^+/CD8^+$ Tregs in 3rd trimester were higher compared with those in non-pregnant women, and the $PD\text{-}1^+/CD8^+$ Tregs in 3rd trimester was higher than that in 1st and 2nd trimester. These data show that PD-1 may play a more important role during late pregnancy. Moreover, we found that $PD\text{-}1^+/CD4^+$ Tregs in 1st trimester RPL women and 3rd trimester GDM women as well as $PD\text{-}1^+/CD8^+$ Tregs in 3rd trimester GDM women were significantly lower compared to the same trimester of normal pregnant women. It is reasonable to assume that a deficiency PD-1 expression might cause the overreaction of T cells, thus play

a role in resulting in RPL and GDM. In addition, PD-1 can induce apoptosis, so it is speculated that the decreased expression of PD-1 may be related to the increased proportion of $CD8^+$ Tregs.

Along with the regulation of Tregs reactivity, GITR induces co-stimulatory signals in $CD4^+$ Tregs and involves in T cell proliferation and cytokine production [54]. High level expression of GITR after activation of $CD8^+$ Tregs is essential for costimulation of $CD8^+$ T cells, because the lack of GITR will reduce the proliferation response of these cells to costimulation of CD28 [21]. The expression of GITR was decreased in repeated implantation failure (RIF) or unexplained RPL [55, 56], which indicates that GITR may play critical roles in regulating the immune response during pregnancy.

In the present study, we found that in normal pregnant women, the proportion of $GITR^+/CD4^+$ Tregs in 3rd trimester and $GITR^+/CD8^+$ in all trimester were higher compared to non-pregnant women. We also found that the proportion of $GITR^+/CD4^+$ Tregs and $GITR^+/CD8^+$ Tregs in 3rd trimester were higher compared with those in 1st and 2nd trimester. These results suggested that the expression of GITR on Tregs vary in different gestational period, and the function of Tregs in immunomodulation and immunosuppression during pregnancy may be different.

The proportion of $GITR^+/CD4^+$ Tregs and $GITR^+/CD8^+$ Tregs in 1st trimester RPL women and 3rd trimester GDM women were significantly decreased compared to non-pregnant women in this study. We also found that $GITR^+/CD4^+$ Tregs in 1st trimester RPL women, $GITR^+/CD4^+$ Tregs and $GITR^+/CD8^+$ Tregs in 3rd trimester GDM women were significantly decreased compared to the same trimester of normal pregnant women, which suggest that deficiency of GITR expression might play a role in the pathogenesis of RPL or GDM.

HLA-G is an immune-modulatory molecule that can inhibit a broad array of immune cells and is strongly involved in fetal-maternal tolerance during pregnancy[57]. HLA-G expressing Tregs has been revealed to inhibit T-cell responses mainly by cell-cell contact independent mechanisms or through secretion of high levels of inhibitory molecules such as IL-10 and soluble HLA-G (sHLA-G) [28, 58]. The development of embryo is closely related to the content of sHLA-G in maternal blood. Therefore, $HLA\text{-}G^+$/Tregs may play an important role in pregnancy as a unique subset. In the present study, we found that the proportion of $HLA\text{-}G^+/CD4^+$ Tregs and HLA-G $^+/CD8^+$ Tregs has no difference during normal pregnancy. However, the proportion of HLA-G $^+/CD8^+$ Tregs in normal pregnant women was significantly higher compared with that in non-pregnant women. These findings suggest that $HLA\text{-}G^+/CD8^+$ Tregs might contribute to establishing an immunosuppressed environment, which allows maternal-fetal tolerance during normal pregnancy. However, the role of $HLA\text{-}G^+$ Tregs in balancing immune responses during pregnancy remains widely elusive and warrants further investigations.

Previous studies reported that $HLA\text{-}G^+/CD4^+$ and $HLA\text{-}G^+/CD8^+$ Tregs were significantly lower in PE women, and HLA-G expression in placenta was decreased in RPL women [59-61]. Our study showed that $HLA\text{-}G^+/CD4^+$ Tregs in 1st trimester RPL and 2nd trimester GDM women as well as $HLA\text{-}G^+/CD8^+$ Tregs in

2nd and 3rd trimester GDM women were lower than those in normal pregnant women. Considering the functions of HLA-G in pregnancy associated immune tolerance, the decreased expression of HLA-G on Tregs in RPL or GDM, at least in part, impair the restraint of undesirable maternal alloreactivity, thus might play a role in pregnancy complications.

CTLA-4 is known to be an important negative regulator of T-cell function. The immunosuppressive functions of Tregs can be influenced by CTLA-4 [62]. Moreover, a significant reduction in the CTLA-4 expression of peripheral blood and decidual tissues from human miscarriage has been previously reported [56, 63, 64]. This study showed that there was no difference in the proportion of CTLA-4^+/ $CD4^+$ Tregs during normal pregnancy and pregnancy complicated by RPL or GDM, which may indicate the decreased contribution of CTLA-4 in $CD4^+$ Tregs suppression during pregnancy.

In the present study, we found that the proportion of CTLA-4^+/$CD8^+$ Tregs in 3rd trimester normal pregnant women and 1st trimester RPL women were higher compared with those in non-pregnant women. These data suggest that CTLA-4 may be important during pregnancy. However, there was no significant difference in the proportion of CTLA-4^+/$CD8^+$ Tregs in RPL and GDM group compared with normal pregnant group.

In our study, we found that the expression of Tregs and surface markers are different in peripheral blood before and during pregnancy. It has been suggested that fetus-specific $CD4^+$ Tregs are specifically recruited from PB to the decidua [65, 66], and our results showed that the increased proportion of $CD4^+$ Tregs in PB might be involved in the maintenance of pregnancy (at the fetal-maternal interface). Additionally, it is more likely that crucial immunological events occur at the fetal-maternal interface, thus future studies are needed to compare Tregs in both the peripheral blood and the decidua from the same normal pregnant women.

In conclusion, the proportion and phenotype of Tregs vary in both normal and compromised human pregnancies. Tregs participate in creating an immunological privileged site for fetus as an allograft. Deregulated PD-1, GITR and HLA-G expressions on Tregs might be implicated in the pathogenesis of RPL and GDM. However, further researches with large sample size are needed for a better understanding of the Tregs immunomodulatory mechanisms in maternal-fetal immune tolerance.

Abbreviations

Tregs:Regulatory T cells; RPL:Recurrent pregnancy loss; GDM:Gestational diabetes mellitus; PD-1:Programmed cell death receptor-1; GITR:Glucocorticoid-inducible TNF receptor family-related protein; HLA-G:Human leucocyte antigen-G; CTLA-4:Cytotoxic T lymphocyte antigen 4.

Declarations

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Authors' contributions

Z.Y.Y. was responsible for the laboratory operation, data analysis and manuscript drafting. L.N. and B.H.C. was responsible for the data acquisition and analysis. Z.X.L.,C.L.J.,C.L.,M.D. and Z.P. were responsible for the specimen collection and data interpretation. N.S. was responsible for the critical discussion and manuscript writing.W.W.J. was responsible for the study design, data analysis and manuscript writing. All authors read and approved the final manuscript.

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Availability of data and materials

The primary data for this study is available from the authors on direct request.

Ethics approval and consent to participate

The study was approved by the Research Ethics Committee of Yantai Yuhuangding Hospital.

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

Publisher's Note

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Tables

Table 1 Demographic and Clinical Characteristics of each group

Item	Non-pregnancy (n=57)	1st trimester normal pregnancy (n=28)	2nd trimester normal pregnancy (n=43)	3rd trimester normal pregnancy (n=33)	1st trimester RPL (n=21)	2nd trimester GDM (n=17)	3rd trimester GDM (n=28)	P
Age (years)	27.88 ± 1.76	26.74 ± 1.18	27.33 ± 1.87	27.45 ± 1.25	27.88 ± 1.76	26.88 ± 1.76	26.49 ± 0.38	0.05
Gestation weeks (Wks)	-	8.58 ± 0.32	26.27 ± 0.24	39.59 ± 0.18	8.45 ± 0.37	26.59 ± 0.18	39.47 ± 0.48	0.05
Birth weight (g)	-	-	-	3329.34 ± 35.62	-	-	3634.66 ± 37.54	0.01
Primiparity								0.05
Yes	-	16 (57%)	20 (46%)	15 (45%)	11 (52%)	9 (52%)	15 (53%)	
No	-	12 (43%)	23 (54%)	18 (55%)	10 (48%)	18 (48%)	13 (47%)	
Fasting glucose (mmol/L)	4.63 ± 0.39	4.66 ± 1.39	4.69 ± 0.87	4.63 ± 0.37	4.69 ± 0.26	4.97 ± 0.76	4.91 ± 0.35	0.05
1-hr plasma glucose after OGTT (mmol/L)	5.52 ± 0.47	5.59 ± 0.73	5.51 ± 1.65	5.46 ± 0.38	5.64±0.46	9.27 ± 0.07	9.13 ± 0.58	0.01

OGTT: oral glucose tolerance test. Data are present as mean ± SEM.

Table 2 Expression of PD-1, GITR, HLA-G and CTLA-4 on $CD4^+$ T cells

Item	Non-pregnancy (n=57)	1st trimester normal pregnancy (n=28)	2nd trimester normal pregnancy (n=43)	3rd trimester normal pregnancy (n=33)	1st trimester RPL (n=21)	2nd trimester GDM (n=17)	3rd trimester GDM (n=28)
PD-1	1.12 ± 0.12	0.74 ± 0.06	0.57 ± 0.07	1.40 ± 0.54	0.75 ± 0.15	0.67 ± 0.12	1.35 ± 0.71
GITR	0.84 ± 0.13	0.68 ± 0.10 d*	0.88 ± 0.14 d*	1.95 ± 0.30a	0.41 ± 0.07 b	0.41 ± 0.09 c	0.91 ± 0.26 d
HLA-G	0.71 ± 0.07	0.45 ± 0.06	0.52 ± 0.05	0.76 ± 0.11	0.28 ± 0.03 b	0.26 ± 0.06 c	0.21 ± 0.14 d*
CTLA-4	0.31 ± 0.04	0.10 ± 0.01	0.26 ± 0.14	0.14 ± 0.02	0.18 ± 0.06	0.07 ± 0.01	0.07 ± 0.02

a vs. Non-pregnancy, P 0.01
b vs. 1st trimester normal pregnancy, P 0.05
c vs. 2nd trimester normal pregnancy, P 0.05
d vs. 3rd trimester normal pregnancy, P 0.05; d*, P 0.01
Data are present as mean ± SEM.

Table 3 Expression of PD-1, GITR, HLA-G and CTLA-4 on $CD4^+$ Tregs

Item	Non-pregnancy (n=57)	1st trimester normal pregnancy (n=28)	2nd trimester normal pregnancy (n=43)	3rd trimester normal pregnancy (n=33)	1st trimester RPL (n=21)	2nd trimester GDM (n=17)	3rd trimester GDM (n=28)
PD-1	2.87 ± 0.33	2.61 ± 0.39 d	2.81 ± 0.40 d	6.29 ± 0.81 a	1.40 ± 0.22 b	1.51 ± 0.32	1.42 ± 1.13 d
GITR	4.24 ± 0.31	3.18 ± 0.39 d	3.94 ± 0.68 d	6.66 ± 0.75 a	1.97 ± 0.36 b	2.05 ± 0.34	2.94 ± 1.31 d
HLA-G	3.37 ± 0.32	2.84 ± 0.39	3.71 ± 0.56	4.12 ± 0.53	1.65 ± 0.28 b	1.54 ± 0.28 c	3.31 ± 0.90
CTLA-4	0.09 ± 0.02	0.22 ± 0.05	0.33 ± 0.10	0.03 ± 0.10	0.21 ± 0.05	0.20 ± 0.08	0.13 ± 0.04

a vs. Non-pregnancy, P □ 0.01
b vs. 1st trimester normal pregnancy, P □ 0.05
c vs. 2nd trimester normal pregnancy. P □ 0.05
d vs. 3rd trimester normal pregnancy, P □ 0.01
Data are present as mean ± SEM.

Table 4 Expression of PD-1, GITR, HLA-G and CTLA-4 on $CD8^+$ Tregs

Item	Non-pregnancy (n=57)	1st trimester normal pregnancy (n=28)	2nd trimester normal pregnancy (n=43)	3rd trimester normal pregnancy (n=33)	1st trimester RPL (n=21)	2nd trimester GDM (n=17)	3rd trimester GDM (n=28)
PD-1	5.35 ± 0.17	10.43 ± 2.86	5.63 ± 1.13	18.74 ± 2.61 abc*	13.11 ± 3.83	6.44 ± 2.56	8.49 ± 2.45 d
GITR	4.61 ± 0.21	14.92 ± 3.23 a	10.94 ± 1.79 a	25.25 ± 3.21 abc*	15.45 ± 3.76	6.69 ± 2.25	8.58 ± 2.56 d*
HLA-G	3.25 ± 0.81	15.71 ± 3.21 a	12.38 ± 1.88 a	16.87 ± 2.13 a	16.09 ± 3.42	5.27 ± 1.81 c	9.01 ± 3.09 d
CTLA-4	1.65 ± 0.67	5.01 ± 1.35	3.95 ± 1.01	6.82 ± 1.62 a	6.74 ± 1.73	2.74 ± 1.56	3.01 ± 1.23

a vs. Non-pregnancy, P □ 0.01
b vs. 1st trimester normal pregnancy, P □ 0.01
c vs. 2nd trimester normal pregnancy, P □ 0.05; c*, P □ 0.01
d vs. 3rd trimester normal pregnancy, P □ 0.05; d*, P □ 0.01
Data are present as mean ± SEM.

Figures

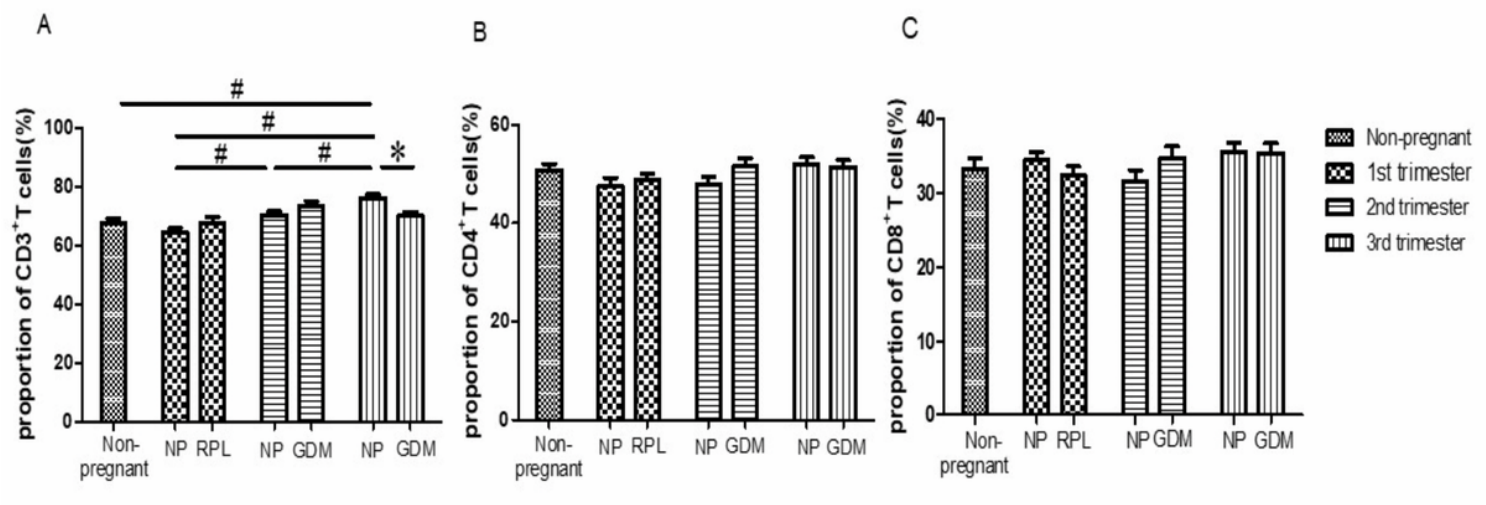

Figure 2

Proportion of CD3+ T cells (A), CD4+ T cells (B), and CD8+ T cells (C) in each group. Data are presented as mean ± SEM. # indicates significant differences by one-way ANOVA followed by Tukey's post-hoc test when compared within different trimester of NP group and non-pregnant group, P 0.01; * indicates significant differences by Student's T-test when compared to NP group, P 0.01.NP: normal pregnant; RPL:recurrent pregnancy loss; GDM: gestational diabetes mellitus.

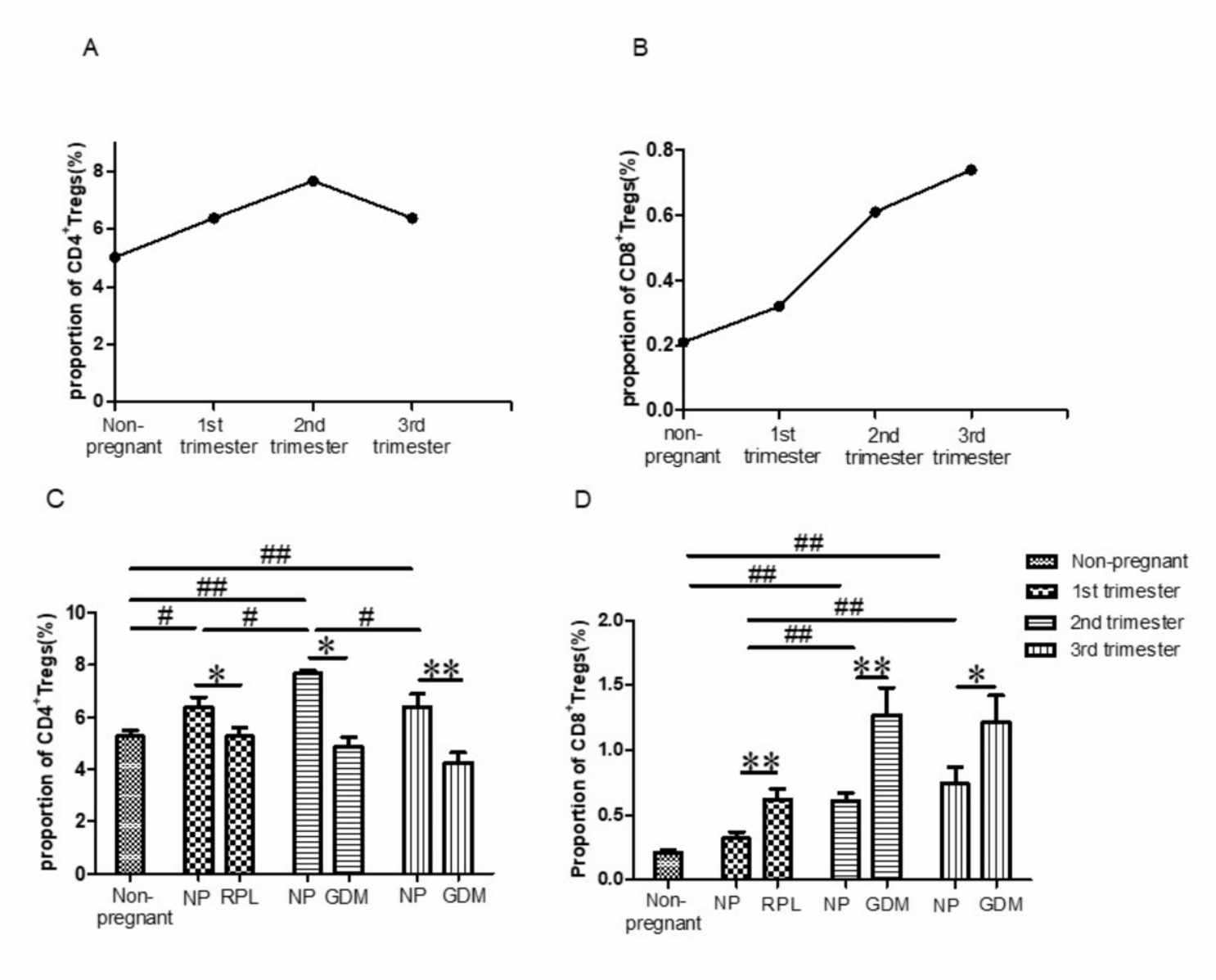

Figure 4

Proportion of CD4+ Tregs and CD8+ Tregs in each group. A. Distribution of CD4+ Tregs in non-pregnant and NP group. B. Distribution of CD8+ Tregs in non-pregnant and NP group. C. Proportion of CD4+ Tregs in non-pregnant, NP, RPL and GDM group. D. Proportion of CD8+ Tregs in non-pregnant, NP, RPL and GDM group. Data are presented as mean ± SEM. # P □ 0.05 and ## P □ 0.01, indicates significant differences by one-way ANOVA followed by Tukey's post-hoc test when compared within different trimester of NP group and non-pregnant group; * P □ 0.05 and ** P □ 0.01, indicates significant differences by Student's T-test when compared to NP group.NP: normal pregnant; RPL:recurrent pregnancy loss; GDM: gestational diabetes mellitus.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- supplementaltable.docx
- supplementaltable.docx