Maternal and Fetal CD4+CD25+CD¹²⁷low/- Regulatory T Cells in Pregnancies with Gestational Diabetes, Preeclampsia, and Premature Rupture of Membranes

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Research

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Abstract

Background Regulatory T cells (Tregs) play a crucial role in maternal-fetal tolerance, but little is known about the characteristic of Tregs in peripheral blood (PB), maternal-fetal interface and cord blood (CB) in normal and complicated pregnancies with preeclampsia (PE), gestational diabetes mellitus (GDM) and premature rupture of membranes (PROM).

Methods PB, retro-placental blood (RPB), and CB were collected immediately after delivery in women with normal full-term pregnancy (NP), PE, GDM, and PROM. The proportion of CD4 + CD25 + CD127 low/- T cells (Tregs) and the expression of PD-1, GITR, HLA-G and CTLA-4 on T cell subsets were investigated by flow cytometric analysis. The data were analyzed based on sample origins (PB, RPB, and CB) and the obstetrical study groups (NP, PE, GDM, and PROM).

Results The proportions of Tregs in PB, RPB, and CB from NP were significantly higher than the other obstetrical groups ($P < 0.01$, respectively). However no significant differences were present among the PE, GDM and PROM groups ($P = \text{NS}$). The proportion of PD-1 + and GITR + Tregs in RPB and PB as well as PD-1 + Tregs in CB from NP were significantly higher than those of the same origin in each obstetrical study group ($P < 0.01$, respectively). There were no differences in HLA-G + and CTLA-4 + Tregs between different origins in each obstetrical study group. In NP, the proportion of PD-1 + Tregs was significantly decreased in CB as compared to those of PB and RPB ($P < 0.05$, respectively). Among the four groups, the proportion of GITR + Tregs were significantly higher in PB as compared to those of CB and RPB ($P < 0.01$, respectively). The proportion of HLA-G + Tregs in PB was significantly lower than that of CB and RPB ($P < 0.01$, respectively). The proportion of CTLA-4 + Tregs had no significant differences ($P = \text{NS}$).

Conclusions In conclusion, the proportion and characteristics of Tregs vary in the maternal, fetal and maternal-fetal junction at the time of delivery. The different feature and density of Tregs in maternal and fetal tissue may suggest the multiple and complicated roles of Tregs during pregnancy.

Background

The maternal immune system has various mechanisms to tolerate semi-allogenic pregnancy. Tregs have been reported to play an essential role in the maintenance of pregnancy. Tregs, identified by the surface markers CD4⁺, CD25⁺, and CD127^{dim/-} [1], are increased in early pregnancy, peaked in mid-pregnancy and decreased in late pregnancy [2]. There are four main processes by which Tregs suppress immune responses: (1) modulation of dendritic cell (DC) function or maturation, (2) release of inhibitory cytokines, (3) cytotoxicity, and (4) metabolic disruption [3]. Accumulating evidence indicated that elevated levels of Tregs are detected in normal pregnancy [4], whereas deficiencies in their quantity and/or function have been demonstrated in obstetrical complications, such as recurrent pregnancy loss (RPL), preeclampsia (PE), gestational diabetes mellitus (GDM) and premature rupture of membrane (PROM) [5-8].

PE, which complicates approximately 10% of pregnancies, is a leading cause of maternal-fetal morbidity and mortality worldwide. There is strong evidence that disturbance in the immune response during early

pregnancy appears to be the central cause of later placental pathology and secondary systemic reaction, such as hypertensive disorder [9-12]. There is substantial evidence that many pregnant women with PE have fewer and less functionally competent Tregs [13, 14]. Therefore, it is conceivable that defective Tregs may be causatively involved in the development of PE. GDM is defined as carbohydrate intolerance with onset or first recognition during pregnancy. Recent evidence indicates that GDM is characterized not only by increased insulin resistance and glucose intolerance but by a state of low-grade systemic inflammation and dysregulation of the immune system, which induces an imbalance of Tregs [15, 16]. Like PE and GDM, the etiology of PROM is multifactorial. However, many studies have focused on the detection of evidence of inflammation during pregnancy [17]. It is generally believed that preterm PROM is causally linked to intra-amniotic inflammation and intra-amniotic infection [18]. Studies have shown that women with intra-amniotic infection had a higher number of total T and CD4⁺ T cells in amniotic fluid [19]. There are phenotypic changes in granulocytes and Tregs, which are consistent with the presence of intravascular inflammation in preterm PROM and preterm labor patients [20, 21]. The inflammatory response is crucial to the onset of labor [22]. Hence, abnormal expression of Tregs could explain the activation of maternal inflammatory cascade, leading to PROM and labor. Since immunosuppressive Tregs were shown to control excessive inflammation, and immoderate immune responses are known to be of vital importance for the successful course of pregnancy, it could be possible that functional deficiencies of such cells are involved in the pathogenesis of PROM.

Diverse expression of surface markers on Tregs may reflect the changes in their function and capacity to expand during pregnancy. There are several critical surface markers on 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). PD-1 is a cell surface receptor belonging to the CD28 family. PD-1 cross-linking with PD-L1 results in the promotion of Tregs development and function [23]. The proportion of PD-1⁺ T lymphocytes were elevated in a normal healthy pregnancy, while a deficiency of PD-1 expression might cause the overreaction of T cells, which occurred in abnormal pregnancies [24-26]. It is reasonable to assume that PD-1 may play critical roles in regulating the immune response during pregnancy.

GITR, which plays inhibitory roles, is constitutively expressed on the cell surface of Tregs. The role of GITR triggering in the maturation/expansion of Tregs has been suggested by previous studies in mice and humans [27, 28]. It is also reported that blockade of GITR ligand reduces both Tregs numbers and immune suppressor phenotype [29-32], which demonstrate the crucial role of GITR in Tregs maturation and expansion. HLA-G, expressed on invasive trophoblast cells, is supposed to confer to maternal-fetal tolerance. Interaction of extra-villous trophoblasts (EVT) with CD4⁺ T cells resulted in increased numbers of CD4⁺CD25^{Hi}Foxp3⁺CD45RA⁺ resting Tregs and increased the expression level of the Treg specific transcription factor Foxp3 in these cells [33, 34]. It has been reported that HLA-G⁺/CD4⁺ T cells were significantly higher in PB from pregnant women [35], while the insufficient expression of HLA-G is correlated with PE and PRL [36, 37]. CTLA-4, which competes with the stimulatory molecule CD28 for CD80 and CD86 binding on antigen-presenting cells, serves an immune-regulatory function on T cell

activation. CTLA-4 is constitutively expressed on Tregs, and its ligation positively reinforces the immunosuppressive functions of Tregs.

Tregs from different origins may have unique phenotypes and various expressions of surface markers. The purpose of this study was to investigate the proportion of Tregs and their phenotypic characteristics in PB, RPB, and CB of normal pregnant women and women with PE, GDM, and PROM.

Methods

Study population

The study was approved by the ethics committee of Yuhuangding hospital, and all study subjects signed informed consent and permission form prior to entering the study. PB was drawn from 94 pregnant women, including 15 PE, 28 GDM, 23 PROM, and 28 NP. 15 CB and RPB were taken from each group separately. Immediately after the cord was clamped, CB and RPB were collected into EDTA-containing tubes for Tregs evaluation. All women with NP delivered vaginally without any complication. None had multiple gestations, pregnancies with chromosomal or fetal abnormalities, diabetes, pregnancy-related hypertension, autoimmune or chronic disease. PE was defined as the occurrence of hypertension; systolic blood pressure of ≥ 140 mm Hg or as the diastolic blood pressure of ≥ 90 mm Hg, plus albuminuria; presence of ≥ 300 mg of protein in 24h urine sample after 20 weeks of gestation in a woman who was normotensive before 20 weeks gestation [11]. The diagnosis of GDM was made between 24 and 28 weeks of gestation by a positive 2-h 75 g oral glucose tolerance test (OGTT) 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) [38]. The diagnosis of PROM was confirmed by the clinical findings of a posterior vaginal pool and the vaginal PH or ferning test. The clinical characteristics of women with normal and complicated pregnancies are presented in Table 1.

Cells preparation and flow cytometry analysis

The following mAbs were used: peridinin chlorophyll protein (PerCP)/Cy5.5-conjugated anti-CD3, fluorescein isothiocyanate FITC-anti-CD4, PE-conjugated anti-CD25, brilliant violet 510 anti-CD127, APC/Fire750 anti-CD279 (PD-1), and PE/Cy7 anti-CD357 (GITR), allophycocyanin APC anti-HLA-G, and brilliant violet421 anti-CD152 (CTLA-4). 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 Tregs was characterized by the gating of CD3⁺, CD4⁺, CD25⁺, and CD127^{low/-} subsets. Then, within these subsets,

PD-1, GITR, HLA-G, or CTLA-4 cells were gated, respectively. The collected data were exported to FlowJo software for analysis (Tree Star, Ashland, OR).

Statistical analysis

Statistical analysis were performed using statistical software GraphPad Prism, version 5 (GraphPad, San Diego, CA, USA). Data were described as mean $\pm$ standard error (SEM). Normality tests were used first to determine if all the data sets are in normal distribution. Student t-test was applied to test the differences between the two groups. ANOVA was used to test the differences between multiple groups. The correlations of the proportion of Tregs with various surface markers between different groups were analyzed by linear correlation analysis. Statistical significance was set at $P < 0.05$.

Results

Clinical characteristics of the study population

The clinical characteristics of the obstetrical study populations, including age, obstetrical history, and outcome of the index pregnancies are listed in Table 1.

The proportions of CD3⁺, CD4⁺, and CD8⁺ T cells in PB, RPB, and CB from NP, PE, GDM, and PROM

The proportion of CD3⁺ T cells in PB was higher compared to RPB and CB in each obstetrical group ($P < 0.01$, respectively). CD3⁺ T cells in each sample origin (PB, RPB, and CB) were the same among obstetrical study groups ($P = \text{NS}$, respectively).

The proportion of CD4⁺ T cells in RPB was lower compared to those of PB and CB in each obstetrical group ($P < 0.01$, respectively). The proportion of CD4⁺ T cells in each sample origin (PB, RPB, and CB) was the same among obstetrical study groups ($P = \text{NS}$, respectively).

The proportions of CD8⁺ T cells in PB, RPB, and CB have no differences in each obstetrical group ($P = \text{NS}$, respectively). The proportions of CD8⁺ T cells in each sample origin (PB, RPB, and CB) were the same among obstetrical study groups ($P = \text{NS}$, respectively) (Table 2)

The proportions of Tregs in PB, RPB, and CB from NP, PE, GDM, and PROM

The proportions of Tregs in PB, RPB, and CB from NP were significantly higher than those of other obstetrical study groups ($P < 0.01$, respectively). In NP, the proportion of Tregs in RPB ($5.25\% \pm 0.33\%$) was significantly lower than those from CB ($6.85\% \pm 0.35\%$) and PB ($7.85\% \pm 0.53\%$) ($P < 0.01$, respectively).

There were no differences in Tregs proportions of each sample origin among PE, GMD, and PROM groups ($P = \text{NS}$, respectively) (Fig. 1A).

The Tregs reduction rates $[(PB-RPB)/PB]$ showed no differences among obstetrical study groups ($P = NS$, respectively) (Fig. 1B).

Expression of PD-1, GITR, HLA-G, and CTLA-4 on CD4⁺ T cells in PB, RPB and CB from NP, PE, GDM and PROM

The proportions of PD-1⁺/CD4⁺ T cells in RPB and PB of NP were significantly higher than those of other obstetrical study groups ($P < 0.01$, respectively). In all obstetrical study groups, the proportions of PD-1⁺/CD4⁺ T cells from RPB were higher than those of CB ($P < 0.01$, respectively) while the proportions of PD-1⁺/CD4⁺ T cells from PB were higher than those of CB ($P < 0.01$, respectively).

In NP, the proportion of GITR⁺/CD4⁺ T cells from PB ($3.01\% \pm 0.58\%$) was higher compared to CB ($0.68\% \pm 0.26\%$) and RPB ($0.91\% \pm 0.28\%$) ($P < 0.01$, respectively). The proportion of GITR⁺/CD4⁺ T cells in PB of NP was significantly higher than those of other obstetrical study groups ($P < 0.01$, respectively).

In all obstetrical study groups, the proportions of HLA-G⁺/CD4⁺ T cells in PB were significantly lower than those of CB ($P < 0.05$, respectively) and RPB ($P < 0.01$, respectively) while the proportions of HLA-G⁺/CD4⁺ T cells in RPB were significantly lower than those of CB ($P < 0.05$, respectively).

The proportions of CTLA-4⁺/CD4⁺ T cells had no statistical differences among the sample origin or obstetrical study groups ($P = NS$, respectively) (Table 3).

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 Tregs in PB, RPB and CB from NP, PE, GDM and PROM

The proportions of PD-1⁺ Tregs in PB, RPB, and CB of NP were significantly higher than those of other obstetrical study groups ($P < 0.01$, respectively). In NP, the proportion of PD-1⁺ Tregs in CB ($6.16\% \pm 0.43\%$) was significantly lower than those of RPB ($8.58\% \pm 0.79\%$) and PB ($8.09\% \pm 0.81\%$) ($P < 0.05$, respectively).

GITR⁺ Tregs in PB and RPB of NP were significantly higher than those of other obstetrical study groups ($P < 0.01$, respectively). In NP, the proportion of GITR⁺ Tregs from PB ($7.71\% \pm 0.87\%$) was significantly higher than those of CB ($0.91\% \pm 0.18\%$) and RPB ($2.51\% \pm 0.63\%$) ($P < 0.01$, respectively). The same findings were present in all obstetrical study groups.

The proportion of HLA-G⁺ Tregs in PB ($2.94\% \pm 0.63\%$) of NP was significantly lower than those of CB ($17.51\% \pm 3.61\%$) and RPB ($25.19\% \pm 4.94\%$) ($P < 0.01$, respectively). The same findings were present in the other obstetrical study groups.

The proportions of CTLA-4⁺ Tregs had no statistical differences among the sample origin or obstetrical study groups ($P = NS$, respectively) (Table 4)

Correlations between Tregs subsets in PB, RPB, and CB from NP

There were significant positive correlations between the proportions of PD-1⁺ Tregs ($r = 0.42$, $P < 0.05$), GITR⁺ Tregs ($r = 0.67$, $P < 0.05$) and HLA-G⁺ Tregs ($r = 0.43$, $P < 0.05$) in CB and RPB. However, there were no significant correlations between the proportions of PD-1⁺ Tregs, GITR⁺ Tregs and HLA-G⁺ Tregs in PB and CB as well as PB and RPB ($P = \text{NS}$, respectively). No significant correlations were present in proportions of CTLA-4⁺ Tregs among the sample origins ($P = \text{NS}$, respectively).

Discussion

Our experimental results showed that the proportions of CD3⁺ T cells from NP, PE, GDM and PROM were significantly higher in PB than in RPB or CB. The proportions of CD4⁺ T cells in RPB from NP, GDM, PE, and PROM were significantly lower than those in PB or CB. Interestingly, there were no differences in CD8⁺ T cells among the sample origins or the obstetrical study groups. The RPB taken immediately after delivery may reflect maternal-fetal interface blood with the potential mixture of maternal blood [39]. We named it as “retro placental blood” in this study. During pregnancy, maternal placental circulation allows bidirectional passage of soluble antigens and nucleated blood cells through the maternal-fetal interface [40]. At the maternal-fetal junction, the fetal blood cells are proximity to the maternal blood, however the mixture of fetal and maternal blood cells does not occur because of the uteroplacental interface [41-43]. In this study, we report that CB retains a lower proportion of CD3⁺ T cells but the same proportion of CD4⁺ T cells with maternal PB. This finding was inconsistent with previous report which demonstrated that CD3⁺ and CD4⁺ T cells were increased in CB compared to PB [44]. More interestingly, our findings showed that CD3⁺ and CD4⁺ T cells in RPB were significantly lower than those in PB, which suggested that T cell-related immunity was suppressed at the maternal-fetal junction at the time of parturition.

CD3⁺ T cells account for 10% of maternal immune cells in the first-trimester human decidua, and about 30-45% of CD3⁺ T cells are CD4⁺ T cells. In contrast, CD3⁺ T cells represent about 80% of lymphocytes in peripheral blood, and approximately 65% of these CD3⁺ T cells are CD4⁺ T cells [45, 46]. These data indicate that CD3⁺ and CD4⁺ T cells are rare in the decidua during early pregnancy, possibly due to a reduced capacity for T cell accumulation [47]. Our findings showed that these cells were also decreased at the maternal-fetal junction during late pregnancy compared to those in PB regardless of obstetrical study groups.

Tregs play a role in modifying the maternal immune response to the fetoplacental ‘allograft’ within the uterus [41, 43, 48, 49]. Previously, we reported that the proportions of Tregs were decreased in PB and decidua of women with unexplained RPL when compared to normal early pregnant women [5, 38]. Contrarily, the temporary elevation of Tregs on the day of embryo transfer was associated with the higher embryo implantation rate [50]. The adoptive transfer of Tregs rescued pregnancy in abortion-prone mice model [51]. Few studies have investigated Tregs at the maternal-fetal junction and CB at the time of delivery [12, 52, 53]. In this study, we found that the proportions of Tregs were significantly decreased in

women with obstetrical complications as compared to NP regardless of sample origins. In addition, the proportions of Tregs in RPB of each obstetrical study group were lower than those in PB in all obstetrical study groups, including NP, and the reduction rates of Tregs were the same among the obstetrical study groups. Hence, the proportion of Tregs in RPB is determined by the proportion of PB Tregs, and the reduction rate of Tregs in RPB was not affected by the presence of obstetrical complications.

Data suggested that fetus-specific Tregs are specifically recruited from PB to the maternal-fetal interface [54]. Recruitment failure of CD4⁺ CD25^{bright} Tregs to the maternal-fetal interface may play a role in the development of obstetrical complications. Both maternal factors and fetal antigenicity were reported to play a role in determining maternal Treg accumulation at the uteroplacental interface [55]. The postpartum decline in Tregs was consistent with the withdrawal of the immunological stimulus of the allograft [2]. In this study, we found that proportions of Tregs in PB, RPB, and CB from PE, GDM and PROM were lower compared to NP, which was consistent with the previous study [56]. It may suggest that immune suppression in the maternal side, as well as the maternal-fetal interface, was less effective in obstetrical complications than those of normal pregnancies.

The characteristics of Tregs, determined by their surface expression of various markers, were different between PB, RPB, and CB in this study. Previous studies showed that PD-1 provided multiple and possibly redundant mechanisms to enhance the suppressive function and stability of Tregs and promote Tregs differentiation [57-59]. While the engagement of GITR on Tregs inhibited their suppressive function and in vivo GITR stimulation increased the numbers of Tregs [60-62]. Our experimental results showed that the proportion of PD-1⁺ and GITR⁺ Tregs were higher in PB and RPB than in CB from NP. Placental or trophoblast-derived soluble factors, including M-CSF, IL-10, TGF- β , and TRAIL may expand and activate Tregs in maternal side but not in fetal side [63]. Maternal PB and RPB contain possibly induced Tregs resulting from an encounter with foreign antigens, while Tregs in CB are functionally mature immune-regulatory population with naive phenotype [64]. Considering the naive phenotype and incomplete post thymic expansion of CB Tregs, the regulatory mechanisms engaged in the augmentation (PD-1 and GITR) of their suppressive activity might be acquired during the postnatal maturation process [65-67]. In this study, we found that the proportion of PD-1⁺/CD4⁺ and GITR⁺/CD4⁺ T cells as well as PD-1⁺ and GITR⁺ Tregs were significantly lower in CB compared to PB and RPB. Besides, the high proportion of PD-1⁺ and GITR⁺ Tregs from PB and RPB suggest that it may create a tolerogenic immune environment that allows the development of allogeneic placental and fetal tissues.

Studies have shown that PD-1⁺ T cells seem to be increased in healthy pregnant women compared to non-pregnant women [68], while decreased PD-1⁺ T cells were observed in PE and GDM[69-71]. In our studies, we found that PD-1⁺ Tregs in CB, PD-1⁺ Tregs and GITR⁺ Tregs in PB from PE, GDM, and PROM were lower compared to those of NP. These findings underscored the critical roles of PD-1 and GITR in regulating Treg function and maintaining immune suppression during pregnancy, whereas insufficient PD-1 and GITR expression might cause unsustainability in maternal-fetal immune tolerance and consequently, lead to pregnancy complications.

The higher proportions of HLA-G⁺ Tregs from RPB and CB in NP, PE, GDM, and PROM are other significant findings of the present study. The expression of HLA-G is confined to immune-privileged sites, although the highest expression is shown in the placenta, where its primary role involves the protection of the fetus from maternal immunity, thus critically contributing to maternal-fetal tolerance [72]. Previous studies demonstrated that the HLA-G⁺/CD4⁺ T cells were expanded in the decidua compared to the periphery [35, 73, 74], which was in line with our results. In this study, we found a much higher proportion of HLA-G⁺ Tregs at the maternal-fetal interface compared to PB. The high density of Tregs might be necessary for fetal acceptance at the site of the highest allogeneic stimulation inevitably presented by the fetus. However, it remains to be clarified whether the HLA-G⁺ Tregs are recruited from the periphery or be induced at the maternal-fetal interface to promote tolerance. Considering the functions of HLA-G in pregnancy-associated immune tolerance, the increased expression of HLA-G on Tregs from RPB and CB may, at least in part, contribute to the restraint of undesirable maternal alloreactivity. HLA-G also stimulates the production of angiogenic factors and cytokines that favor embryo implantation, placental vascularization, and maternal-fetal tolerance [75]. Low blood concentrations of soluble HLA-G (sHLA-G) have been associated with increased risks of PE, GDM, and intrauterine growth restriction [76-78]. In this study, the proportion of HLA-G⁺ Tregs in all sample origins of NP tends to be higher than those of PE, GDM and PROM, however the differences did not reach a statistically significant level. It may be due to small sample size, and further study is needed.

CTLA-4 is accepted as a crucial negative regulator of T-cell responses. Upon the stimulation of the TCR, CTLA-4 becomes stabilized on the surface of T cells, thus competing with CD28 for B7 binding and impairs the activation of T cells. It is reported that the expression of CTLA-4 on the cell surface is a key for Tregs to exert suppression by a critical mechanism [79-82]. In animal studies, treatment with CTLA-4-blocking antibody caused greater susceptibility to fetal loss with altered cytokine profiles by decidual CD4⁺ T (dCD4⁺ T) cells [83]. It is also reported that percentages of CTLA-4⁺ cells were significantly increased in the endometrium of women with recurrent implantation failure (RIF) and RPL than those in controls [84]. This study showed that there was no difference in the proportions of CTLA-4⁺ Tregs between the obstetrical study groups, which may indicate the decreased contribution of CTLA-4 in Tregs suppression during pregnancy.

In conclusion, Tregs possess different immunologic features in terms of phenotype and function in PB, RPB, and CB. Deregulated PD-1 and GITR expressions on Tregs may be associated with obstetrical complications, such as PE, GDM and PROM. HLA-G⁺ Tregs in RPB may serve for the maternal-fetal tolerance during pregnancy. Further studies are needed to investigate the maternal factors and microenvironment, which affect the development and function of Tregs during pregnancy.

Abbreviations

Tregs: Regulatory T cells; PB: Peripheral blood; RPB: Retro-placental blood; CB: Cord blood; NP: Normal full-term pregnant; PE: Preeclampsia; GDM: Gestational diabetes mellitus; PROM: Premature rupture of

membranes; 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. Z.X.L. and S.H. was responsible for the data acquisition and analysis. C.L. and M.D. were responsible for the specimen collection, data interpretation and critical discussion. J.K.K and 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.

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Tables

Table 1 Age, obstetrical history and outcome of the index pregnancies from NP women,women with PE, GDM or PROM

Items	NP (n = 28)	PE (n = 15)	GDM (n = 28)	PROM (n = 23)	P
Age (years)	26.74 ± 1.18	28.33 ± 1.87	27.45 ± 1.25	27.88 ± 1.76	NS
Gestation weeks (Wks)	39.58 ± 0.32	39.75 ± 0.24	39.59 ± 0.18	39.45 ± 0.37	NS
Birth weight (g)	3329.34 ± 35.62	2837.57 ± 32.68	3634.66 ± 37.54	3415.56 ± 36.59	< 0.01
Primiparity					
Yes	16(57%)	7 (46%)	15(53%)	11 (47%)	NS
No	12 (43%)	8(54%)	13(47%)	12 (53%)	
Mode of Delivery					
Vaginal delivery	28(100%)	0	10(35%)	12(52%)	< 0.01
Cesarean section	0	15(100%)	18(65%)	11(48%)	
Blood pressure (mmHg)	≤ 120/80	≥ 140/90	≤ 120/80	≤ 120/80	< 0.01
Proteinuria (mg/dL/day)	≤ 150	≥ 300	≤ 150	≤ 150	< 0.01
Fasting glucose (mg/dl)	85.76 ± 1.39	86.37 ± 0.87	89.48 ± 0.76	86.23 ± 1.26	< 0.01
1-hr plasma glucose after OGTT (mg/dl)	100.67 ± 0.73	99.25 ± 1.65	169.99 ± 1.07	101.53 ± 1.46	< 0.01

OGTT:oral glucose tolerance test, NS:no significance

Table 2 Proportion of CD3⁺, CD4⁺, and CD8⁺ T cells in PB, RPB and CB from NP women, women with PE, GDM or PROM

Items(%)		NP (n = 28)	PE (n = 15)	GDM (n = 28)	PROM (n = 23)	P ^a
CD3 ⁺ T cells	PB	76.77 ± 1.62 ^{b, c}	75.37 ± 1.69 ^{b, c}	75.46 ± 1.54 ^{b, c}	76.29 ± 1.78 ^{b, c}	NS
	RPB	63.75 ± 1.01	63.73 ± 1.34	63.36 ± 1.42	64.27 ± 1.27	NS
	CB	63.13 ± 1.87	62.78 ± 1.24	62.25 ± 1.34	63.33 ± 1.35	NS
	P ^d	< 0.01	< 0.01	< 0.01	< 0.01	
CD4 ⁺ T cells	PB	46.62 ± 1.17	46.25 ± 1.42 ^b	48.48 ± 1.33	47.55 ± 1.39 ^b	NS
	RPB	38.45 ± 1.33 ^{c, e}	37.79 ± 1.54 ^{c, e}	39.28 ± 1.57 ^{c, e}	36.36 ± 1.11 ^{c, e}	NS
	CB	50.34 ± 1.67	51.55 ± 1.58	51.66 ± 1.17	52.27 ± 1.54	NS
	P ^d	< 0.01	< 0.01	< 0.01	< 0.01	
CD8 ⁺ T cells	PB	25.44 ± 1.28	25.73 ± 1.43	26.4 ± 1.32	27.32 ± 1.41	NS
	RPB	26.38 ± 1.50	26.34 ± 1.45	25.30 ± 1.83	26.43 ± 1.58	NS
	CB	24.56 ± 1.40	24.25 ± 1.47	24.29 ± 1.33	25.55 ± 1.41	NS
	P ^d	NS	NS	NS	NS	

^a Comparison with NP, PE, GDM and PROM by ANOVA.

^b indicates $P < 0.01$ when compared to PB and CB in the same obstetrical group.

^c indicates $P < 0.01$ when compared to PB and RPB in the same obstetrical group.

^d Comparison with PB, RPB and CB in the same obstetrical group by ANOVA.

^e indicates $P < 0.01$ when compared to RPB and CB in the same obstetrical group.

Values are mean ± SEM. NS:no significance

Table 3 Expression of PD-1, GITR, HLA-G and CTLA-4 on CD4⁺ T cells in PB, RPB and CB from from NP women, women with PE, GDM or PROM

Items(%)		NP (n = 28)	PE (n = 15)	GDM (n = 28)	PROM (n = 23)	P ^a
PD-1	PB	5.85 ± 1.08 ^{b**}	2.69 ± 0.15 ^{b**, c}	2.54 ± 0.71 ^{b**, c}	2.74 ± 0.12 ^{b**, c}	< 0.01
	RPB	7.29 ± 1.63 ^{d**}	4.47 ± 0.42 ^{d**, c}	4.29 ± 0.53 ^{d**, c}	4.52 ± 0.33 ^{d**, c}	< 0.01
	CB	0.43 ± 0.07	0.15 ± 0.37	0.37 ± 0.46	0.23 ± 0.69	NS
	P ^e	< 0.01	< 0.01	< 0.01	< 0.01	
GITR	PB	3.01 ± 0.58 ^{b**}	0.65 ± 0.09 ^c	0.9 ± 0.26 ^c	0.55 ± 0.10 ^c	< 0.01
	RPB	0.91 ± 0.28 ^f	0.86 ± 0.39	0.83 ± 0.58	0.73 ± 0.85	NS
	CB	0.68 ± 0.26	0.77 ± 0.25	0.76 ± 0.33	0.64 ± 0.36	NS
	P ^e	< 0.01	< 0.01	< 0.01	< 0.01	
HLA-G	PB	0.55 ± 0.09 ^{b*, f}	0.21 ± 0.04 ^{b*, f}	0.21 ± 0.14 ^{b*, f}	0.31 ± 0.05 ^{b*, f}	NS
	RPB	16.88 ± 3.18 ^{d*}	14.28 ± 3.54 ^{d*}	15.24 ± 4.72 ^{d*}	14.27 ± 4.24 ^{d*}	NS
	CB	7.05 ± 2.42	5.62 ± 1.79	6.35 ± 2.79	5.73 ± 1.28	NS
	P ^e	< 0.01	< 0.01	< 0.01	< 0.01	
CTLA-4	PB	0.07 ± 0.04	0.06 ± 0.05	0.07 ± 0.02	0.07 ± 0.03	NS
	RPB	0.08 ± 0.04	0.07 ± 0.11	0.05 ± 0.03	0.05 ± 0.08	NS
	CB	0.07 ± 0.02	0.05 ± 0.03	0.06 ± 0.04	0.07 ± 0.02	NS
	P ^e	NS	NS	NS	NS	

^a Comparison with NP, PE, GDM and PROM by ANOVA.

^{b*} indicates $P < 0.05$, ^{b**} indicates $P < 0.01$ when compared to PB and CB in the same obstetrical group.

^c indicates $P < 0.01$ when compared to NP.

^{d*} indicates $P < 0.05$, ^{d**} indicates $P < 0.01$ when compared to RPB and CB.

^e Comparison with PB, RPB and CB by ANOVA.

^f indicates $P < 0.01$ when compared to RPB and CB in the same obstetrical group.

Values are mean $\pm$ SEM. NS:no significance

Table 4 Expression of PD-1, GITR, HLA-G and CTLA-4 on Tregs in PB, RPB and CB from from NP women, women with PE, GDM or PROM

Tregs(%)		NP (n = 28)	PE (n = 15)	GDM (n = 28)	PROM (n = 23)	P ^a
PD-1	PB	8.09 $\pm$ 0.81	1.86 $\pm$ 0.26 ^b	1.42 $\pm$ 1.13 ^b	1.49 $\pm$ 0.28 ^b	< 0.01
	RPB	8.58 $\pm$ 0.79 ^c	1.82 $\pm$ 0.38 ^b	1.33 $\pm$ 0.27 ^b	1.33 $\pm$ 0.27 ^b	< 0.01
	CB	6.16 $\pm$ 0.43 ^{d*}	1.79 $\pm$ 0.01 ^b	1.45 $\pm$ 0.25 ^b	1.38 $\pm$ 0.48 ^b	< 0.01
	P ^e	< 0.05	NS	NS	NS	
GITR	PB	7.71 $\pm$ 0.87	3.07 $\pm$ 0.41 ^b	2.94 $\pm$ 1.3 ^b	2.84 $\pm$ 0.33 ^b	< 0.01
	RPB	2.51 $\pm$ 0.63 ^f	0.28 $\pm$ 0.27 ^{b, f}	0.26 $\pm$ 0.27 ^{b, f}	0.27 $\pm$ 0.28 ^{b, f}	< 0.01
	CB	0.91 $\pm$ 0.18 ^{d**}	0.47 $\pm$ 0.25 ^{d**}	0.36 $\pm$ 0.25 ^{d**}	0.62 $\pm$ 1.15 ^{d**}	NS
	P ^e	< 0.01	< 0.01	< 0.01	< 0.01	
HLA-G	PB	2.94 $\pm$ 0.63 ^{d**}	1.36 $\pm$ 0.25 ^{d**}	3.31 $\pm$ 0.90 ^{d**}	1.47 $\pm$ 0.23 ^{d**}	NS
	RPB	25.19 $\pm$ 4.94 ^f	27.37 $\pm$ 5.47 ^f	26.46 $\pm$ 4.73 ^f	26.46 $\pm$ 5.11 ^f	NS
	CB	17.51 $\pm$ 3.61	15.46 $\pm$ 4.67	16.14 $\pm$ 4.67	16.46 $\pm$ 4.16	NS
	P ^e	< 0.01	< 0.01	< 0.01	< 0.01	
CTLA-4	PB	0.12 $\pm$ 0.03	0.11 $\pm$ 0.01	0.13 $\pm$ 0.04	0.12 $\pm$ 0.05	NS
	RPB	0.12 $\pm$ 0.11	0.11 $\pm$ 0.25	0.13 $\pm$ 0.25	0.12 $\pm$ 0.27	NS
	CB	0.11 $\pm$ 0.02	0.14 $\pm$ 0.33	0.12 $\pm$ 0.24	0.12 $\pm$ 0.14	NS
	P ^e	NS	NS	NS	NS	□

^a Comparison with NP, PE, GDM and PROM by ANOVA.

^b indicates $P < 0.01$ when compared to NP.

^c indicates $P < 0.05$ when compared to RPB and CB.

^{d*} indicates $P < 0.05$, ^{d**} indicates $P < 0.01$ when compared to PB and CB.

^e Comparison with PB, RPB and CB by ANOVA.

^f indicates $P < 0.01$ when compared to PB and RPB in the same obstetrical group.

Values are mean $\pm$ SEM. NS:no significance

Figures

Figure 2

Proportion of Tregs in PB, RPB and CB(A) and the reduction rate of Tregs(B) from NP women or women with GDM, PE and PROM. Values are mean $\pm$ SEM. *indicates significant differences from NP by Student's T-test, $P < 0.01$. # indicates significant differences by one-way ANOVA followed by Tukey's post-hoc test indicates when compared to RPB $P < 0.05$. ## indicates significant differences by one-way ANOVA followed by Tukey's post-hoc test indicates when compared to RPB $P < 0.01$

Supplementary Files

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

- [SupplementalTable.docx](#)
- [SupplementalTable.docx](#)