

Full Research Paper

Changes in the Ratio of Tc1/Tc2 and Th1/Th2 Cells but Not in Subtypes of NK-Cells in Preeclampsia

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Abstract: It has been suggested that natural killer (NK) cell activity and Th1 immunity may be involved in the pathogenesis of preeclampsia. This study aimed to investigate the immunophenotypes of NK cells and type 1/type 2 immunity in both decidua and maternal peripheral blood between normal (n=11) and preeclamptic pregnant women (n=20) by flow cytometry. The results showed that no significant difference was observed between patients and controls by detecting CD56⁺CD69⁺ and CD56⁺CD94⁺ NK cells in both peripheral blood and decidua. Moreover, in preeclamptic patients, decreased percentages of Tc2 and Th2 cells and the increased ratios of Tc1/Tc2 were determined in both decidua and maternal peripheral blood. In addition, the ratio of Th1/Th2 in peripheral blood also increased. There was no significant difference of immunophenotypes of uNK cells between preeclampsia and normal pregnancy. Local decidua and systematic immunity did not correlate with each other. These results suggest that the type 1/type 2 immunity shifted to type 1 immunity including Th1 and Tc1 cells may contribute to the patho-genesis of preeclampsia.

Keywords: Decidua, uNK cell, NK cell, type 1/type 2 immunity, preeclampsia, human

1. Introduction

Preeclampsia is the leading cause of pregnancy-related maternal and fetal morbidity and mortality. The pathogenesis of this disorder remains obscure. Its earliest pathologic change is in the uteroplacental circulation, with decreased penetration and dilatation of the spiral arteries. Immune maladaptation may cause shallow invasion of spiral arteries. NK cells comprise over 70 % of endometrial leukocytes in first trimester decidua. Decidual NK cells have a diverse role in pregnancy, including regulating excessive trophoblast invasion into the deciduas and formation of uterine spiral arteries after implantation. Some evidences show that dysfunctional natural killer (NK) cell activation may be related to the pathogenesis of preeclampsia [1-4]. The percentages of NK cells in the decidua and the umbilical cord blood of the preeclamptic patients were changed [5,6]. Moreover, some studies show there are two distinct subsets of NK cells, defined as CD56^{dim}CD16⁺ and CD56^{bright}CD16⁻ NK cells [7]. CD56^{bright}CD16⁻ NK cells have prominent cytoplasmic granules and are minimally cytotoxic, while CD56^{dim}CD16⁺ NK cells are more cytotoxic [8]. However it is unclear whether the percentage of these subsets was changed in preeclampsia.

Except for CD56 and CD16, human NK cells also express several members of the C-type lectin family, such as CD69 and CD94. CD69 is considered as an activation marker on activated NK to induce cytotoxic activity. CD94 expresses on a majority of CD56^{bright} uterine NK (uNK) cells as an inhibition marker, and may play an important role in maintaining pregnancy [9]. Ntrivalas et al. and Thum et al. found that elevated expression of CD69 and decreased expression of CD94 on NK cells might cause placental dysfunction or implantation failure [10-12]. Preeclampsia is related to trophoblast invasion [13], so it is necessary to investigate the expression of CD69 and CD94 on NK cells or NK cells subsets in preeclampsia.

Many authors believe that an imbalance of the Th1/Th2 type responses, with a shift towards a Th1 response, may be involved in the etiology of preeclampsia [14], but the results on the Th1/Th2 in preeclampsia were inconsistent with each other. An elevated plasma level of Th2 type cytokines such as IL-4 was observed in preeclamptic patients [15]. No difference was found in Th1-cytokine production in peripheral blood and fetal cord blood between preeclamptic patients and normal pregnancies [16,17]. In addition, CD8⁺ T (Tc) cells can differentiate into type 1 (Tc1) cells, producing mainly IFN- γ , and type 2 (Tc2) cells, producing mostly IL-4, IL-5, and IL-10. The Tc1/Tc2 balance also can modulate the type 1/type 2 immunity [18]. The increased decidual Tc lymphocyte percentage was detected in preeclamptic pregnancies [19], but the ratio of Tc1/Tc2 is still unclear in preeclampsia.

The decidua is the maternal tissue in closest contact with the fetal tissue. It may form a local immunity to maintain pregnancy [20]. However, changes between the peripheral blood systemic and the decidua local immunity may be not parallel. Previous studies mainly focused on the immune changes in maternal peripheral blood or decidua separately [5,15-17]. Therefore, for the present study, the maternal peripheral blood and decidua from healthy and preeclamptic pregnant women were obtained. The ratios of NK cells expressed CD56, CD16, CD69 or CD94, as well as Th1/Th2 and Tc1/Tc2 were detected. The interrelation of these ratios was compared between normal and preeclamptic pregnant women.

2. Materials and methods

2.1. Inclusion criteria

Preeclampsia was diagnosed and classified according to the criteria recommended by the 21st edition of "Williams Obstetrics". The study groups were selected from among the pregnant women hospitalized in the Affiliated Drum Tower Hospital, Medical School of Nanjing University from September 2005 to March 2006. Through interviews, clinical investigations and laboratory tests, the absence of diabetes, renal diseases, chronic hypertension, intrahepatic cholestasis of pregnancy, hyperthyroidism, hypothyroidism or symptomatic infectious diseases in the participating subjects was ensured. The selected pregnant women presented no uterine contractions, premature rupture of membranes or clinical chorioamnionitis complications. The selected pregnant women qualified for elective caesarean sections in this study.

2.2. Group characteristics

Some parameters identified from interviews, clinical examination, and laboratory tests are showed in Table 1.

Table 1. Parameters from healthy pregnant women (control) group and three groups with preeclampsia.

Parameters	Control(n=11)	MP(n=9)	SP(n=11)	TP(n=20)
Patient age (year)	25.2±2.5	26.8±1.7	28.2±4.2	27.6±3.3
Gestational age (weeks)	39.9±1.1	38.8±1.3*	35.6±2.5**	37.0±2.6**
Mean systolic pressure (mm Hg)	114±10.0	141.3±5.8**	162.1±8.5**	152.8±12.8**
Mean diastolic pressure (mm Hg)	64.0±19.9	90.6±5.22*	108.5±8.2**	96.4±23.4**
Proteinuria (dispstick)	-	1+	2+-4+	1+-4+
Birth weight of fetus (g)	3645±336.6	3402.2±455.7	2302.3±575.5**	2797.3±759.4**

Compared with the controls * P < 0.05 and ** P < 0.01. MP = mild preeclampsia; SP= severe preeclampsia; TP = total preeclampsia.

2.3 Cell isolation

Two ml of maternal peripheral blood were taken from each patient and healthy woman and collected in sterile heparinized tubes. Decidua tissue (about 1 g) was obtained by curettage from the maternal-fetal interface and then placed into bottles containing sterile phosphate-buffered saline (PBS). The

decidual slices were rinsed with PBS to remove residual blood, torn fragments, and then digested by Collagenase IV for 30 minutes at 37 °C [21,22]. The peripheral blood and decidual mononuclear cells were both obtained by Ficoll-Hypaque gradient centrifugation.

2.4. Immunophenotypic Analysis

Two-color immunofluorescence staining was used to analyze NK cell immunophenotypes from decidua and peripheral blood. CD56 (MEM-188; phycoerythrin (PE)) was used to identify NK cells and uNK cells. CD16 (3G8; fluorescein isothiocyanate (FITC)), CD69 (CH/4; FITC) or CD94 (DX22; FITC) were used to determine the subsets of NK cells. Anti-CD56 and anti-CD94 monoclonal antibodies were purchased from eBioscience Corporation, USA. Anti-CD16 and anti-CD69 monoclonal antibodies were from Caltag Laboratories, USA.

Cells were stained with the monoclonal antibodies at a density of 10^6 cells/ml in PBS (100 μ l/sample) and incubated for 30 minutes at 4 °C in the dark. Following two washes with PBS, cells were resuspended and fixed with 1 % paraformaldehyde. The cells were then analyzed by flow cytometer (Becton Dickinson FACS). Triggering was set on the forward scatter channel, and the threshold was adjusted to exclude debris. Leucogate was used to measure the proportion of lymphocytes in the sample. Directly labeled isotype control antibodies were used for the exclusion of nonspecific binding. 10000 Events were acquired in the gate. The data was then analyzed using the CellQuest software.

2.5. Type 1/type 2 immunity analyses

A portion of the isolated decidual and peripheral blood mononuclear cells was suspended in RPMI 1640 supplement with 10 % fetal serum, 10 nM phorbol-12 myristate-13 acetate (PMA), 3 μ M monensin and 1 μ M ionomycin (all reagents from Alexis Corporation, UK). The cells were incubated at 37 °C, 5 % CO₂ for 4 h and then stained with CD3 (S4.1; phycoerythrin-cytochrome 5) and CD8 (3B5; FITC) for 30 min at 4 °C in the dark. Following two washes with PBS, the cells were resuspended and fixed with 4% paraformaldehyde for 20 min. The cells were washed again and suspended in 0.1 % saponin buffer for 20 min and incubated with anti-IFN- γ (B27; PE) or anti-IL-4 (MP4-25D2; PE) for 30 min. The cell population was detected by the flow cytometer and analyzed by CellQuest software. The assay system was considered to be reliable and reproducible [23]. Anti-CD3, anti-CD8, anti-IFN- γ and anti-IL-4 antibodies were all from Caltag Laboratories, USA.

2.6. Statistical Analysis

All data were presented as mean \pm SD. Data were analyzed by the Wilcoxon signed rank test, a nonparametric paired *t*-test, the Mann-Whitney U-test and the Pearson correlation test, with $p < 0.05$ considered significant.

2.7 Ethics

This study was approved by the Ethics Committee at the affiliated Drum Tower Hospital of Medical School of Nanjing University and the informed consent was given by all participants in the study.

3. Results

3.1. NK cells subsets in decidua and peripheral blood from preeclamptic patients and the controls

Representative immunophenotypes of uNK cells and NK cells are presented in Figure 1. No differences were detected in the percentages of uNK cells subsets between preeclampsia and normal pregnancies ($p > 0.05$). Data is shown in Table 2, Figure 2.

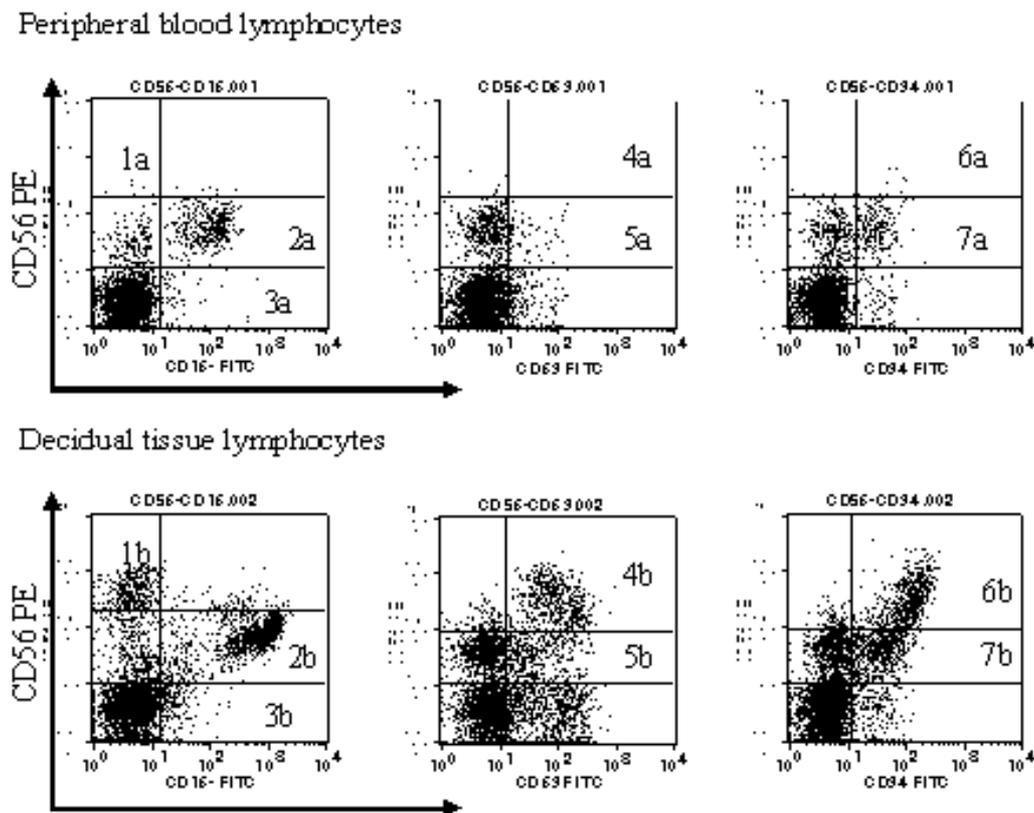


Figure 1. Two-color flow cytometric analysis of immunophenotypes of NK cells in normal term pregnancy. a, peripheral blood. b, deciduas. 1) $CD56^{\text{bright}}CD16^-$; 2) $CD56^{\text{dim}}CD16^+$; 4) $CD56^{\text{bright}}CD69^+$; 5) $CD56^{\text{dim}}CD69^+$; 6) $CD56^{\text{bright}}CD94^+$; 7) $CD56^{\text{dim}}CD94^+$. Percentages of these cell populations within the lymphocytes were: 1a, 0.2%. 1b, 7.15%. 2a, 12.13%. 2b, 27.4%. 4a, 0.01%. 4b, 7.17%. 5a, 3.09%. 5b, 12.39%. 6a, 0.24%. 6b, 8.59%. 7a, 7.79%. 7b, 17.54% respectively.

The percentages of uNK cell subsets in decidua were different from those in peripheral blood. The percentages of $CD56^{\text{bright}}CD16^-$ uNK cell subsets were higher than those in peripheral blood ($p < 0.01$), while the percentages of $CD56^{\text{dim}}CD16^+$ uNK cells subsets had no significant difference

between peripheral blood and decidua in normal term pregnancy ($p > 0.05$). But in preeclampsia, the percentages of $CD56^{dim}CD16^+$ NK cells subsets in peripheral blood were also higher than those in decidua ($p < 0.05$). And the proportions of uNK cell subsets in decidua did not correlate with those in peripheral blood ($p > 0.05$). Data is shown in Table 2.

Table 2. Percentages of NK cells and uNK cells subsets in lymphocytes from preeclampsia patients and control.

Parameters	Mean(\pm SD) proportion (%) of lymphocytes				NK cells subsets were compared and correlated between B and D			
		C(n=11)	MP(n=9)	SP(n=11)	TP(n=20)	P	r	
	$CD56^{bright}CD16^-$	B	0.26 \pm 0.18	0.26 \pm 0.14	0.31 \pm 0.15	0.29 \pm 0.15	C	0.003
	D	17.91 \pm 16.75	16.86 \pm 8.66	17.67 \pm 13.23	17.30 \pm 11.12	TP	<0.001	0.303
$CD56^{dim}CD16^+$	B	16.93 \pm 7.78	20.97 \pm 3.90	23.27 \pm 9.54	22.23 \pm 7.46	C	NS	0.511
	D	16.23 \pm 8.77	18.16 \pm 8.9	14.83 \pm 8.62	16.33 \pm 8.69	TP	0.025	0.144

Compared with the controls * $P < 0.05$; C = control group; MP = mild preeclampsia; SP = severe preeclampsia; TP = total preeclampsia; B = peripheral blood; D = decidua; P shows the difference of NK cells subsets between peripheral blood and decidua in TP or C; r shows the correlation coefficients of NK cells subsets between peripheral blood and decidua in TP or C.

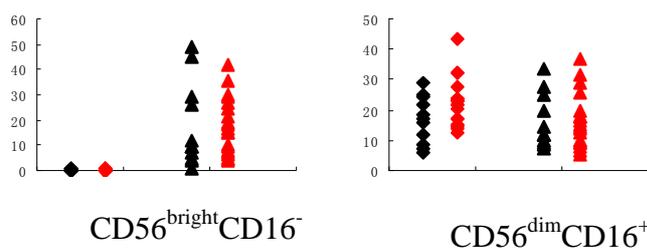


Figure 2. Individual percentages of NK cell subsets in lymphocytes from preeclamptic patients and the controls. Rhombus, in peripheral blood. triangle, in deciduas. Black, the controls (n = 11). Red, the preeclamptic patients (n = 20).

3.2. *CD69 and CD94 expression on decidua and peripheral blood NK cells from preeclamptic patients and the controls*

Compared with control, no difference was shown in the percentages of $CD56^+CD69^+$ and $CD56^+CD94^+$ NK cells and the ratio of $CD56^+CD69^+/CD56^+CD94^+$ in both peripheral blood and decidua of preeclamptic patients. Data is shown in Table 3, Figure 3. The percentages of $CD56^+CD69^+$,

CD56⁺CD94⁺ and CD56^{bright}CD94⁺ uNK and the ratio of CD56⁺CD69⁺/CD56⁺CD94⁺ were higher in decidua than the corresponding peripheral blood (p < 0.01). Data is shown in Table 3.

Table 3. Percentages of NK cells receptors: CD69 and CD94 in lymphocytes and ratios of CD69/CD94 in preeclampsia patients and the controls.

Parameters	Mean(± SD)proportion (%) of lymphocytes				CD69 and CD94 on NK cells were compared and correlated between B and D			
	C(n=11)	MP(n=9)	SP(n=11)	TP(n=20)	D	P	r	
CD56 ⁺ CD69 ⁺	B	3.29±1.21	2.63±0.96	3.38±1.66	3.04±1.41	C	0.003	0.157
	D	36.79±19.7	39.21±18.19	36.86±20.28	37.92±18.90	TP	<0.001	0.018
CD56 ⁺ CD94 ⁺	B	13.43±6.83	17.00±4.23	15.41±7.15	16.13±5.93	C	0.004	0.283
	D	32.71±16.17	37.54±11.70	32.68±17.77	34.87±15.17	TP	<0.001	0.260
CD69/CD94	B	0.29±0.12	0.16±0.07	0.28±0.17	0.23±0.14	C	0.003	0.114
	D	1.21±0.63	1.01±0.27	1.13±0.10	1.08±0.20	TP	<0.001	0.109

Compared with the controls * P < 0.05; C= control group; MP= mild preeclampsia; SP= severe preeclampsia; TP= total preeclampsia; B= peripheral blood; D= deciduas. P shows the difference of expression of CD69 and CD94 on NK cells between peripheral blood and decidua in TP or C. r shows the correlation coefficients of expression of CD69 and CD94 on NK cells between peripheral blood and decidua in TP or C. CD69/CD94 = CD56⁺CD69⁺ %/CD56⁺CD94⁺ %.

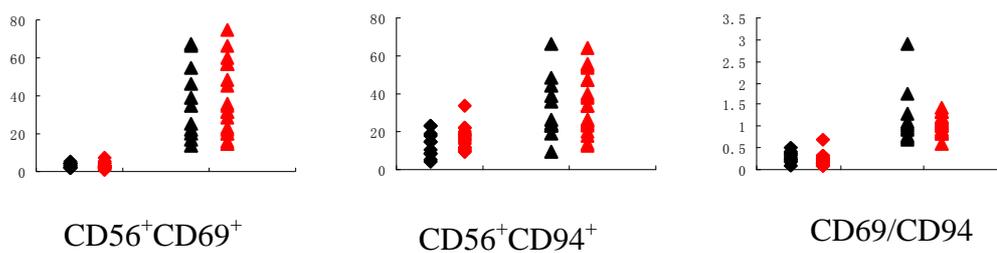


Figure 3. Individual percentages of NK cell receptors: CD69 and CD94 in lymphocytes and ratios of CD69/CD94 in preeclamptic patients and the controls. Rhombus, in blood. triangles, in deciduas. Black , the controls (n=11). Red , the preeclamptic patients (n = 20).

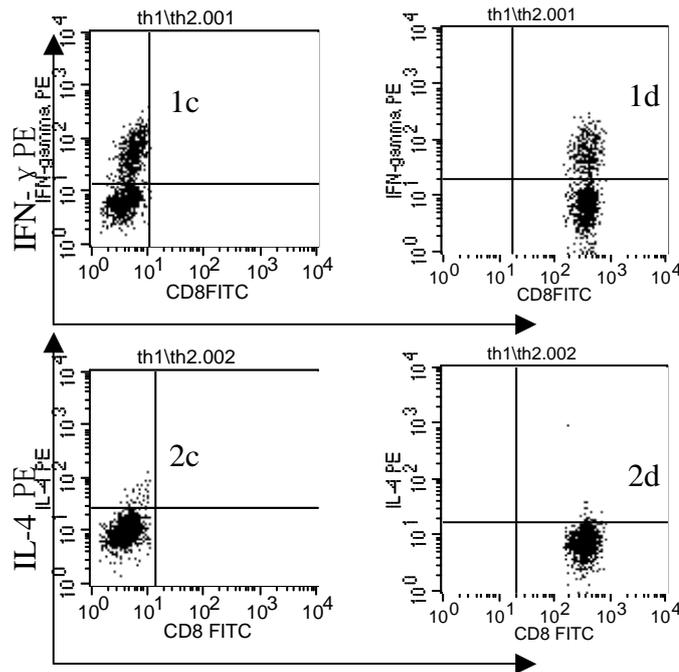


Figure 4. Three-color flow cytometric analysis of type 1/type 2 immunity in blood from normal term pregnancy. **1c, Th1 cells. 2c, Th2 cells. 1d, Tc1 cells. 2d, Tc2 cells.** Percentages of these cell populations were: 1c, 38.92 %. 2c, 3.58 %. 1d, 48.85 %. 2d, 3.38 %.

Table 4. Percentages of Th1, Th2, Tc1, Tc2 and ratios of Th1/ Th2, Tc1/ Tc2 in preeclampsia patients and the controls.

Parameters	Mean(±SD)proportion(%) of positive T cells				Type1/type2 immunity were compared and correlated between B and D			
		C(n=11)	MP(n=9)	SP(n=11)	TP(n=20)	P	r	
Th1	B	31.44±10.74	28.45±11.59	25.37±4.61	26.75±8.38	C	NS	0.445
	D	37.85±9.55	37.39±11.17	36.89±9.59	37.11±10.05	TP	0.004	0.061
Th2	B	2.93±1.03	2.23±0.81	1.91±0.99*	2.05±0.91*	C	NS	0.487
	D	2.94±0.98	2.40±0.75	2.62±0.82	2.52±0.78	TP	0.004	0.618**
Tc1	B	36.16±16.9	39.76±11.88	31.42±9.44	35.17±11.15	C	NS	0.471
	D	47.49±14.92	51.83±13.00	44.51±13.77	47.81±13.60	TP	0.006	0.059
Tc2	B	3.10±1.33	2.49±1.20	2.17±0.44*	2.31±0.86*	C	NS	0.389
	D	4.32±0.92	2.80±0.75**	3.09±1.1*	2.96±0.95**	TP	0.008	0.054

Compare with the controls * P < 0.05, ** P < 0.01; C = control group; MP = mild preeclampsia; SP = severe preeclampsia; TP = total preeclampsia; B = peripheral blood; D = deciduas. P shows the difference of type 1/type 2 immunity between peripheral blood and decidua in TP or C. r shows the correlation coefficients of type 1/type 2 immunity between peripheral blood and decidua in TP or C. Th1=IFN-γ⁺CD8⁻CD3⁺/CD8⁻CD3⁺(%), Th2=IL-4⁺CD8⁻CD3⁺/CD8⁻CD3⁺(%), Tc1=IFN-γ⁺CD8⁺CD3⁺/CD8⁺CD3⁺(%), Tc2=IL-4⁺CD8⁺CD3⁺/CD8⁺CD3⁺(%).

3.3. Type 1/type 2 immunity in decidua and peripheral blood from preeclamptic patients and the controls

Representative intracellular cytokines of T cells are presented in Figure 4. In order to investigate type 1/type2 immunity, CD3⁺CD8⁻(Th) and CD3⁺CD8⁺ T (Tc) cells-producing IFN- γ or IL-4 in vitro were measured. Tc includes IFN- γ -producing CD3⁺CD8⁺ T (Tc1) and IL-4-producing CD3⁺CD8⁺ T (Tc2). In preeclamptic patients, decreased percentages of Tc2 and Th2 cells were observed both in decidua and maternal peripheral blood ($p < 0.05$). In normal term pregnancy, no significant differences in the percentages of Th1, Tc1, Th 2 or Tc2 cells was shown between decidua and peripheral blood, but in preeclamptic patients, the percentages of Th1,Th2,Tc1 and Tc2 cells were higher in decidua than peripheral blood ($p < 0.05$). Data is shown in Table 4, Figure 5.

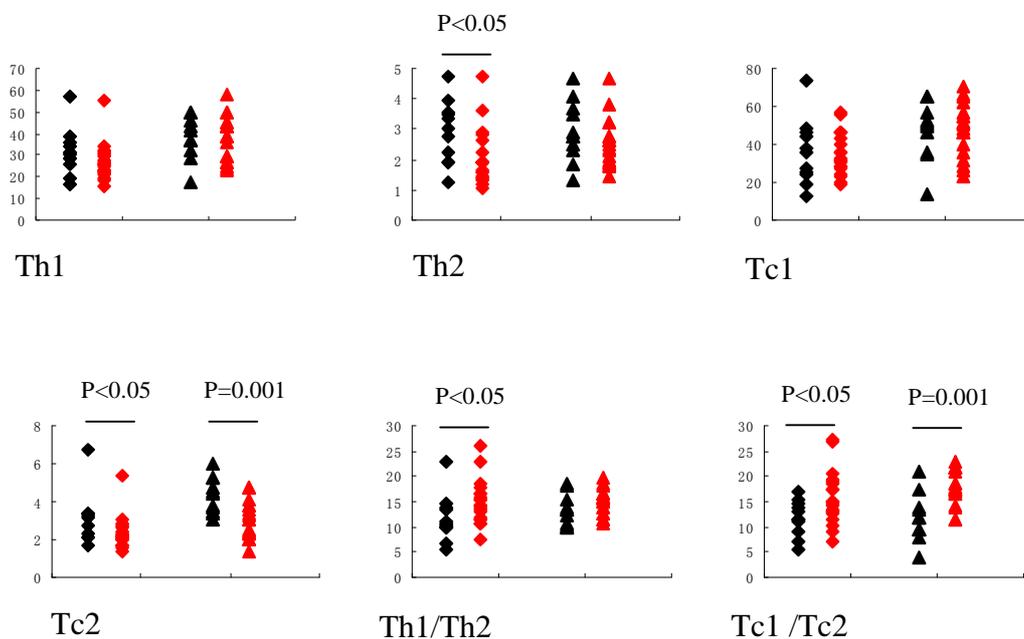


Figure 5. Individual percentages of Th1, Th2, Tc1 and Tc2 and ratios of Th1/ Th2 and Tc1/ Tc2 in preeclamptic patients and the controls. Rhombus, in blood. triangle, in deciduas. Black, the controls (n=11). Red, the preeclamptic patients (n=20).

4. Discussion

In the third trimester pregnancies the percentages of NK cells subsets were different in decidua than in maternal peripheral blood. This result is consistent with previous reports [5, 24]. Moreover, the present results showed that the percentages of CD56^{bright}CD16⁻ and CD56^{dim}CD16⁺ uNK cell subsets in decidua lymphocytes were about 18 % and 16 % respectively, and the percentage of CD56^{bright}CD16⁻ uNK cells was significantly higher than that in peripheral blood (Table 2). Some research has shown that the CD56^{bright}CD16⁻ uNK subset was recruited from maternal peripheral blood to decidua [25], but the differences on the percentages were not reported in previous studies. We speculate that

CD56^{bright}CD16⁻ uNK cells may play an important role in regulating trophoblast invasion and spiral artery transformation, but the correlation of proportions of uNK subsets in maternal peripheral blood with decidua was not observed. Furthermore, there were no significant difference between the patients and controls. Therefore, it is still needed to clarify how and why CD56^{bright}CD16⁻ uNK cells are accumulated in the maternal-fetal interface.

No difference was shown in the percentages of CD56⁺CD69⁺ and CD56⁺CD94⁺ NK cells and the ratio of CD56⁺CD69⁺/CD56⁺CD94⁺ in both peripheral blood and decidua of preeclamptic patients. Some studies demonstrated that the imbalance of CD69/CD94 on NK cells could cause placental dysfunction or implantation failure, and preeclampsia was secondary to an anomaly of the invasion of trophoblast, so we expected to find an imbalance of CD69/CD94 on NK cells in preeclamptic patients. However, no significant difference was obtained in peripheral blood and decidua between preeclamptic patients and normal term pregnancies. It is possible that CD69-mediated NK cytotoxicity may be abrogated by inhibitory receptor CD94 so as to keep the balance of CD69⁺/CD94⁺ in these preeclamptic patients [26, 27]. On the other hand, uNK cells, especially the CD56^{bright}uNK cells, express much more CD69 than those in peripheral blood. By the third trimester, about 36% of lymphocytes were CD56⁺CD69⁺ uNK cells in decidua and the ratio of CD56⁺CD69⁺/CD56⁺CD94⁺ was 1.2 (Table 3). This may reflect the uNK cells were activated in the maternal-fetal interface [10, 28].

Decreased percentages of Tc2 and Th2 cells were observed both in decidua and maternal peripheral blood in preeclamptic patients. Type 1 immunity could hamper immunological tolerance of preeclamptic women to the foreign antigens of the fetus. Recent data demonstrated that up-regulation of Th1 response was not only in peripheral blood but also in placenta of preeclamptic patients [29, 30], but there were also many contrary results [15-17,31]. The lack of consistency may be attributed to the relatively short half-life of the cytokines and the differences in used methods and specimens. In this study, we simultaneously obtained the specimens both from decidua and peripheral blood, and used sensitive flow cytometry to determine the intracellular IFN- γ and IL-4 in Th and Tc cells. We found that the proportions of Th2 cells in peripheral blood and Tc2 cells in decidua were significantly less in preeclampsia than in the controls. So the ratios of Tc1/ Tc2 both in decidua and peripheral blood, and the ratio of Th1/Th2 in peripheral blood of the preeclamptic patients were significantly changed. Th1 and Tc1 cells are type 1 immunity cells, and Th2 and Tc2 cells are type 2 immunity cells [32]. The imbalance of Tc1/Tc2 may contribute to the imbalance of type 1/type 2 immunity. The imbalance in both maternal peripheral blood and decidua may exacerbate preeclampsia. Interestingly, in the preeclamptic patients, the percentages of Th1, Th2, Tc1 and Tc2 cells were significantly higher in decidua than in peripheral blood. This may be that T cells in decidua of preeclamptic patients have been activated by the foreign antigens from the fetus.

In conclusion, the type 1/type 2 immunity shifted to type 1 immunity including Th1 and Tc1 cells that may contribute to the pathogenesis of preeclampsia. There were no significant difference of immunophenotypes of uNK between preeclampsia and normal pregnancy. Local decidua and systematic immunity did not correlate with each other.

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