Circulating and Decidual Th17 Cell Levels in Healthy Pregnancy

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Problem
The Th1/Th2 paradigm has recently been reconstituted to include a third population, Th17 cells. It has been reported that Th2 type immunity is predominantly present in normal pregnancy. However, the level of Th17 cells during pregnancy is still unclear. We investigated the level of peripheral Th17 cells in healthy pregnancy subjects.

Method of study
To evaluate the levels of Th17 cells, we investigated the proportion of peripheral blood mononuclear cells that produced IL-17 in the first, second, and third trimester pregnancy subjects using flow cytometry. We further studied the proportion of decidual lymphocytes that produced IL-17 in early pregnant subjects.

Results
Most of the IL-17-producing cells were $CD4^+$ T cells. The number of circulating Th17 cells did not change during pregnancy. In a paired *t*-test of early normal pregnant subjects, the proportion of IL-17^+ decidual lymphocytes was significantly higher than that of peripheral blood lymphocytes.

Conclusion
Th17 levels in peripheral blood lymphocytes do not change during normal pregnancy.

Introduction

Wegmann et al. proposed that Th2 cytokines may play an important role in the maintenance of murine pregnancy by inhibiting Th1 responses that induce pregnancy failure.[1] His hypothesis has since been adopted in the maintenance of normal human pregnancy,[2,3] pregnancy failure such as recurrent spontaneous abortion,[4,5] and pre-eclampsia.[6,7] However, recent data on cytokine expression at the materno–fetal interface indicated some problems with this hypothesis.[8] The Th1/Th2 paradigm has recently been reconstituted to include a third population of T helper cells that produce IL-17, which are designated as Th17 cells.[9–11] Th17 cells have specific roles in host defense against extracellular bacteria and fungi[12] and play an important role in the induction of autoimmune diseases.[9–11] Recent data revealed that transforming growth factor (TGF)-β is able to induce the differentiation of regulatory T (Treg) cells and that the combination of the pro-inflammatory cytokine IL-6 and TGF-β is able to induce the differentiation of Th17 cells from naïve T cells *in vitro* in mice,[13] although the processes behind the induction and regulation of Th17 cells in humans are different from those in mice.[11] Recent data show that Treg cells play a very important role in the maintenance of pregnancy,[14,15] and decreased numbers of Treg cells have been reported in abortion and pre-eclampsia.[16–18] An imbalance between Th17

and Treg cells has been proposed to occur in case of human diseases such as autoimmune diseases and transplant rejection.[19,20] However, no such imbalance has been reported for Th17 levels during pregnancy.

In this study, we measured the proportions of IL-17-producing cells within circulating and decidual $CD4^+$ T cells during pregnancy.

Materials and methods

Sample Collection and the Isolation of Lymphocytes

Informed written consent was obtained from all patients included in this study. All of the tissue sampling methods and uses described in this study were approved by the Toyama University Ethics Committee. All patients were Japanese.

Heparinized venous blood samples were obtained from 11 non-pregnant women, 30 first trimester pregnant subjects, 10 s trimester pregnancy subjects, and 12 third trimester pregnancy subjects. Almost all of the blood sampling of the non-pregnant subjects was performed at the secretory phase. These patients were selected from women with regular menstrual cycles of 26–31 days. Peripheral blood mononuclear cells were isolated using the standard Ficoll–Hypaque method. Clinical details were recorded for each woman. The clinical indexes except body mass index (BMI) and gestational age were matched among these four groups (Table I).

For analysis of decidual and peripheral blood lymphocytes, 12 specimens were obtained from patients who had undergone elective termination of pregnancy (maternal age median: 28 years, range: 20–38 years; gestational age median: 7 weeks, range: 6–9 weeks). Decidual mononuclear cells (leukocytes) were purified using the Ficoll–Hypaque method after homogenization and filtration through a 32-μm nylon mesh.[15] Decidual tissues were not enzymatically digested so as to prevent the possibility that enzymatic treatment would affect the fluorescence intensity of surface antigens. All groups were subject to the same exclusion criteria: women with infectious, autoimmune, or other systemic or local diseases were excluded.

Flow Cytometry

Peripheral blood mononuclear cells (PBMCs) were stimulated with phorbol myristate acetate (PMA; 10 ng/mL, Sigma Chemical Co., Deisenhofen, Germany) and 2 μg/mL of ionomycin (Sigma Chemical Co.) in the presence of 10 μg/mL of brefeldin A (Sigma Chemical Co.) for 4 hr at 37°C in an atmosphere containing 5% CO_2. Decidual mononuclear leukocytes were also stimulated with PMA

Table I Characteristics of Non-Pregnant, 1st, 2nd, and 3rd Trimester Pregnant Women

		Pregnancy		
	Non-pregnancy	1st trimester	2nd trimester	3rd trimester
n	11	30	10	12
Age (years)	32 (26–37)	28 (17–46)	31 (28–40)	31 (26–39)
Gravidity	1 (0–2)	1 (0–4)	1 (0–4)	1.5 (0–2)
Parity	1 (0–2)	1 (0–3)	0 (0–3)	1 (0–2)
Gestational age (weeks)[a]	–	7 (4–11)	23 (15–25)	35 (29–36)
BMI	21.5 (17.1–25.1)	21.1 (16.1–27.0)	22.6 (18.2–27.3)	24.0 (19.3–29.1)

Data are expressed as median (range).
Numbers of gravidity excluded the pregnancy of this study.
BMI, body mass index.
[a]Gestational age at blood sampling. *P*-values: age (Non versus lst: $P = 0.16$, Non versus 2nd: $P = 0.48$, Non versus 3rd: $P = 0.99$, 1st versus 2nd:$P = 0.09$, 1st versus 3rd:$P = 0.13$, 2nd versus 3rd: $P = 0.46$); Gravidity (Non versus lst: $P = 0.93$, Non versus 2nd: $P = 0.60$, Non versus 3rd: $P = 0.96$ 1st versus 2nd: $P = 0.49$, 1st versus 3rd: $P = 0.97$, 2nd versus 3rd: $P = 0.56$); Parity (Non versus lst: $P = 0.57$, Non versus 2nd: $P = 0.76$, Non versus 3rd: $P = 0.72$ 1st versus 2nd: $P = 0.38$, 1st versus 3rd: $P = 0.85$, 2nd versus 3rd: $P = 0.53$); Gest. age (1st versus 2nd, 1st versus 3rd, 2nd versus 3rd:all $P < 0.001$); BMI (Non versus lst: $P = 0.75$, Non versus 2nd: $P = 0.21$, Non versus 3rd: $P = 0.007$ 1st versus 2nd: $P = 0.20$, 1st versus 3rd: $P = 0.002$, 2nd versus 3rd: $P = 0.19$).

(5 ng/mL) and ionomycin (1 μg/mL) in the presence of brefeldin A (10 μg/mL) for 4 hr at 37°C in an atmosphere containing 5% CO_2. These mononuclear cells were stained for 20 min at room temperature with FITC-conjugated mAb to CD4, CD8, or CD14, respectively (BD Pharmingen™, San Diego, CA, USA). The cells were washed and fixed in 4% formaldehyde/PBS for 5 min at room temperature, before being treated with permeabilizing solution buffer (BD Bioscience, SanJose, CA, USA) for 10 min at room temperature. They were then stained with PE-conjugated anti-IL-17 (eBioscience, San Diego, CA, USA) for 30 min on ice. After being washed, the cells were analyzed on a FACS Calibur flow cytometer using CellQuest software (BD Bioscience). We counted 50,000 cells in each sample. A gate was set to separate PBMC and decidual mononuclear leukocytes using characteristic forward (FSC) and side (SCC) scatter parameters. Monocyte and lymphocyte populations were divided by manual gating (Fig. 1a, upper panels). Intracellular cytokine patterns were analyzed using flow cytometry. The analyses of CD4 and CD8 staining were performed using the cells obtained from the lymphocyte-gated PBMC or decidual mononuclear cells, and the analysis of CD14 was carried out using the cells obtained from the monocyte-gated PBMC (Fig. 1a, upper panels). An isotype-matched PE-conjugated mouse IgG1 antibody (eBioscience) was used as a control.

Statistical Analysis

Background data are presented as the median value and range. *P*-values less than 0.05 were considered significant. The frequency of IL-17 cells in $CD4^+$ T cells was analyzed using the ANOVA test. In comparisons between decidual and peripheral IL-17 ratios, the paired *t*-test was used. The analyses of age, gravidity, parity, gestational age, and BMI were performed with the unpaired *t*-test.

Results

The Proportion of IL-17 Positive Cells During Pregnancy

We first elucidated the main IL-17-producing subset of peripheral lymphocytes. Fig. 1a shows the flow cytometric profiles of IL-17-producing cells for peripheral blood lymphocytes and decidual lymphocytes. The expression of IL-17 was mainly detected in $CD4^+$ T cells in both peripheral lymphocytes and

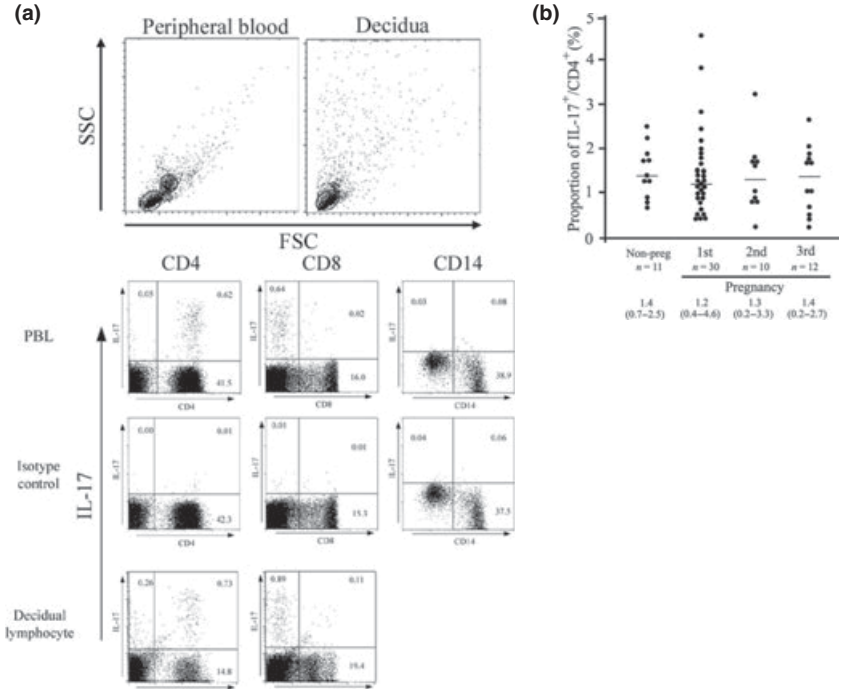

Fig. 1 IL-17 expression in peripheral blood lymphocytes: (a) Upper two panels: FSC is shown on the *x*-axis and SSC is shown on the *y*-axis. The lower gate indicates the lymphocyte and the upper gate indicates the monocytes in peripheral blood (left panel). The gate in decidua indicates the lymphocytes (right panel). Lower nine panels: The intensity of IL-17 staining is shown on the *y*-axis, whereas the intensity of $CD4^+$ (left panels), $CD8^+$ (middle panels), or $CD14^+$ (right panels) staining is plotted on the *x*-axis. The numbers represent the percentages of dots in each gated area. PBL (upper panels), PBL treated with isotype control for PE-conjugated anti-IL-17 antibody (middle panels), and decidual lymphocytes (lower panels) were used. (b) The ratios of IL-17^+ to $CD4^+$ cells in the peripheral blood lymphocytes of non-pregnant and 1st, 2nd, and 3rd trimester pregnant women. The bars indicate median values. The numbers in the lower rows represent the median value and range.

decidual lymphocytes. IL-17$^+$ cells were few in CD8$^+$ T cells or CD14$^+$ monocytes in peripheral lymphocytes (Fig. 1a, second row). Subsequently, we analyzed the ratio of IL-17$^+$ to CD4$^+$ cells in the peripheral blood lymphocytes of normal pregnant women. The median values and ranges of the ratio of IL-17$^+$ to CD4$^+$ cells were 1.2% (0.4–4.6), 1.3% (0.2–3.3), and 1.4% (0.2–2.7) in the first, second, and third trimester normal pregnant women, respectively (Fig. 1b); whereas, the peripheral lymphocytes of non-pregnant women showed a IL-17$^+$ to CD4$^+$ cell ratio of 1.4% (0.7–2.5) (Fig. 1b). No significant differences were detected in the proportion of IL-17± cells among these groups (*P*-values: Non versus 1st: $P = 0.81$, Non versus 2nd: $P = 0.92$, Non versus 3rd: $P = 0.96$, 1st versus 2nd: $P = 0.84$, 1st versus 3rd: $P = 0.78$, 2nd versus 3rd: $P = 0.98$). Furthermore, we compared the ratio of IL-17$^+$ to CD8$^+$ cells in normal pregnant women during pregnancy. The median values and ranges of the ratio of IL-17$^+$ to CD8$^+$ were 0.1% (0–0.7), 0.1% (0–0.5), and 0% (0–1.4) in the first, second, and third trimester normal pregnant women, respectively. There were no significant differences among these groups. Thus, the levels of IL-17$^+$ cells remained stable in peripheral lymphocytes before and during pregnancy.

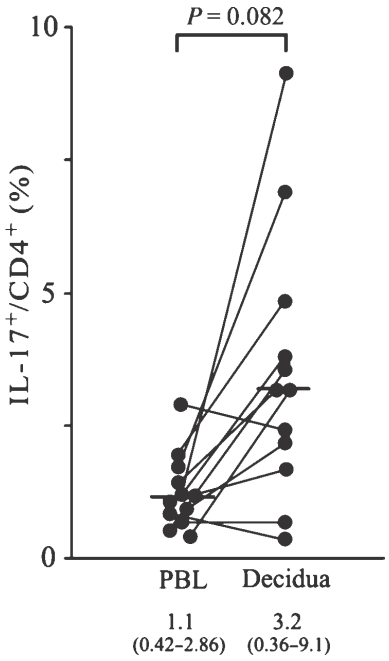

Fig. 2 Comparison of the ratio of IL-17$^+$ to CD4$^+$ cells between the peripheral blood and decidual lymphocytes in 1st trimester pregnant women. The ratios of IL-17$^+$ to CD4$^+$ cells in peripheral blood lymphocytes (PBL: left) and decidual lymphocytes (Decidua: right) are shown. A paired *t*-test was performed. The bars indicate median values. The numbers represent the median value and range.

Comparison of IL-17 Positive Cell Rates Between Peripheral Blood and Decidual Lymphocytes

As mentioned above, the ratio of IL-17$^+$ to CD4$^+$ cells remained stable in peripheral blood lymphocytes during pregnancy. As the next step, we compared the IL-17 ratios between peripheral blood and decidual lymphocytes in the first trimester pregnant women. The median values and ranges of IL-17$^+$ cells were 1.1% (0.4–2.9) and 3.2% (0.4–9.1) in peripheral blood and decidual lymphocytes, respectively (Fig. 2). The ratio of IL-17± to CD4± cells in decidual lymphocytes was significantly higher than that in peripheral blood lymphocytes ($P \leq 0.01$). In four of 12 paired samples, the ratios of IL-17$^+$ to CD4$^+$ cells in decidual lymphocytes were stable, compared with those in the peripheral blood. However, there was no difference between the four samples and the other eight samples with regard to their clinical data. These results indicated that Th17 cells represent a higher proportion of lymphocytes in human decidua compared with that in the peripheral blood in the first trimester.

Discussion

Th2-polarizing immunity is observed in normal pregnancy,[2,3] whereas a shift in Th1/Th2 balance to Th1-polarizing immunity is seen in complicated pregnancies such as those involving abortion and preeclampsia.[4–7] A new unique subpopulation of CD4$^+$ T cells, Th17 cells, may influence the tolerance system during pregnancy. Elevation in IL-17 mRNA and IL-17 protein was observed in an acute renal rejection model,[21,22] and neutralization of IL-17 prevented acute rejection of aortic and cardiac allografts.[23] These data suggest that the proportion of Th17 cells might be decreased during pregnancy to prevent rejection. However, our study revealed that the Th17 cell population remained very stable from the first pregnancy period to the late pregnancy period.

Th17 cells are formed in response to the production of TGF-β and IL-6 produced by dendritic cells; whereas, TGF-β in the absence of IL-6 promotes the differentiation of naïve T helper cells into Foxp3$^+$ Treg cells in mice.[13] However, the pathway of Th17 differentiation in humans is different from that in

mice. IL-1β and IL-6, but not TGF-β, are essential for the differentiation of human Th17 cells.[24]

McClain et al. reported that mice immunized with neuroantigen during pregnancy showed a reduced incidence of experimental autoimmune encephalomyelitis as well as reduced clinical severity.[25] They also showed that immunized pregnant mice produced less IL-17 and TNF-α, suggesting that Th17 cells might decrease in number during pregnancy. Ostojic et al. reported that IL-17$^+$ cells were localized in the glands and in the basal proliferative stroma at days 6.5, 8.5, 9.5, and 10.5 in mice.[26] By day 12.5, the decidua was totally negative for IL-17 staining. This study suggests that the number of Th17 cells is decreased after day 12.5 at the feto–maternal interface in mice. However, our results showed that the ratio of IL-17$^+$ cells was increased in decidual lymphocytes in the first trimester, compared with that in peripheral blood lymphocytes. We did not obtain exactly the same results in humans as that found in mice; i.e. the population of peripheral blood Th17 cells did not change during pregnancy. Serum levels of IL-17 do not change in normal pregnancy or pre-eclampsia.[27] Th17 levels might not change in pre-eclampsia, although Th1 type immunity is predominantly observed in pre-eclampsia.[6,7] Additionally, as shown in Fig. 2, we observed that the proportions of CD4$^+$ T cells that were IL-17$^+$ remained stable between PBL and decidual lymphocytes in four cases of 12 paired samples. Unknown immunologic factors may affect the population of Th17 cells in humans.

Th17 cells play a pivotal role in the induction of the neutrophil-mediated protective immune response against extracellular bacteria and fungal pathogens.[10,13] To prevent these infections, Th17 levels must be kept stable during pregnancy. However, we have recently found that the amniotic fluid IL-17 levels in chorioamnionitis complicated preterm labor cases were significantly higher than those in pregnancies without chorioamnionitis.[28] Therefore, the number of IL-17 cells might be increased in cases of chorioamniotic infection, and IL-17 might participate in host defense.

Choriocarcinoma-derived JEG-3 cell culture supernatant reduces IL-17 and IFN-γ production in mixed-lymphocytes reactions.[29] On the contrary, immunostaining for IL-17 was observed in term placental trophoblasts,[30] and IL-17 was found to induce an increased invasive capacity in JEG cells.[31] However, in our study, we did not observe IL-17 staining in early or term placental trophoblasts. Further studies are needed to elucidate the effects of IL-17 on successful and complicated pregnancies in future.

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