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

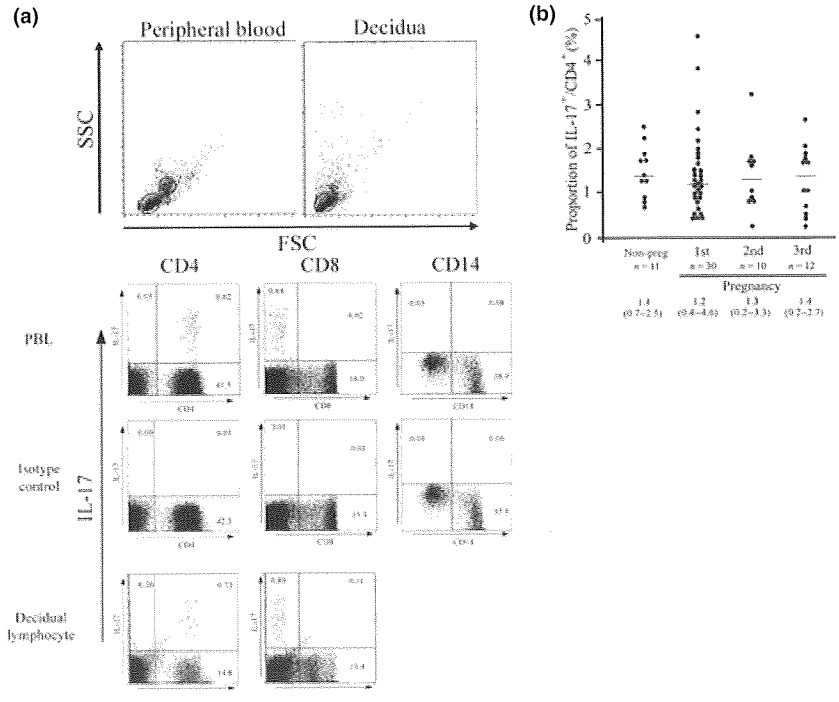

Fig. 1 IL-17 expression in peripheral blood lymphocytes: (a) Upper two panels: FSC is shown on the *x*-axis and SSC is shown on the *y*-axis. The lower gate indicates the lymphocyte and the upper gate indicates the monocytes in peripheral blood (left panel). The gate in decidua indicates the lymphocytes (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 $IL\text{-}17^+$ 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.

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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.

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.

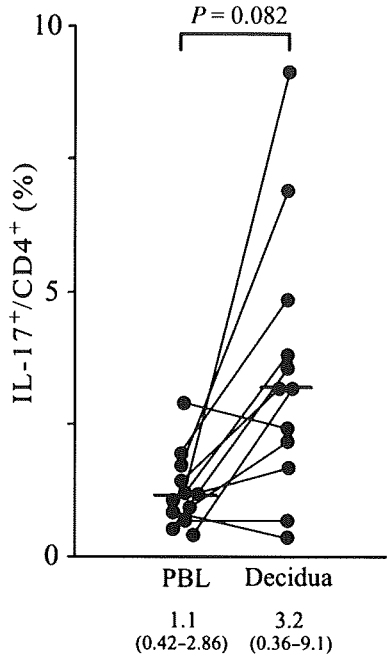

Fig. 2 Comparison of the ratio of IL-17$^+$ to CD4$^+$ cells between the peripheral blood and decidual lymphocytes in 1st trimester pregnant women. The ratios of IL-17$^+$ to CD4$^+$ cells in peripheral blood lymphocytes (PBL: left) and decidual lymphocytes (Decidua: right) are shown. A paired *t*-test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

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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Accumulation of IL-17-Positive Cells in Decidua of Inevitable Abortion Cases

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Keywords
Decidua, inflammation, miscarriage, Th17

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Problem
Th17 cells, a new subset of helper T cells, have been focused on as a producer pro-inflammatory cytokines. It is, however, still unknown how Th17 cells affect pregnancy outcome. We investigated the expression of IL-17-producing cells in human spontaneous abortion.

Method of study
IL-17 expression was analyzed in decidual tissues among normal pregnancy, missed abortion, and inevitable abortion cases by immunohistochemistry and flow cytometry.

Results
IL-17$^+$ cells were accumulated in decidua and were detected in decidual CD4$^+$ T cells and few decidual CD8$^+$ T cells in spontaneous abortion cases. The number of decidual IL-17$^+$ cells in inevitable abortion cases involving active genital bleeding was significantly higher than that in normal pregnancy cases ($P < 0.05$). On the other hand, there were no significant differences in the numbers of decidual IL-17$^+$ cells between missed abortion cases and normal pregnancy subjects. Furthermore, the number of IL-17$^+$ cells was positively correlated with the number of neutrophils in spontaneous abortion cases.

Conclusion
IL-17$^+$ cells might be involved in the induction of inflammation in the late stage of abortion, but not in the early stage of abortion.

Introduction

CD4$^+$ helper T cells are classified as T-helper (Th) 1 cells or Th2 cells according to their patterns of cytokine production.[1] Th1 cells produce interleukin (IL)-2, interferon (IFN)-γ, and tumor necrosis factor (TNF)-α, and they are presumed to cause spontaneous abortion,[2,3] although conflicting data have also been reported.[4,5] Recently, a novel family of CD4$^+$Th cells was detected, which was characterized by IL-17 production and named 'Th17'.[6,7] IL-17, a pro-inflammatory cytokine, induces the expression of many mediators of inflammation. So far, experimental autoimmune encephalomyelitis (EAE) and collagen-induced arthritis are believed to be Th1 response-related diseases, but recent data have shown that Th17 cells play a central role in the pathogenesis of these diseases.[8] Interestingly, the differentiation and functions of Th17 cells and regulatory T (Treg) cells occur in opposite directions. The differentiation of Th17 cells is initiated by transforming growth factor (TGF)-β1 and IL-6, which activate signal transducer and activator of transcription 3 (Stat3) and induce the expression of the transcription factor retinoic acid-related orphan receptor gamma t (RORγt). On the other hand, the presence of TGF-β1 but not IL-6

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induces the expression of Foxp3, resulting in Treg induction.[9] It is well known that Treg cells play very important roles in the maintenance of allogeneic pregnancy,[10] and decreased numbers of Treg cells and decreased expression of Foxp3 mRNA are observed in the decidua and endometrium in abortion[11] and implantation failure.[12] An elevation in IL-17 was also observed in an acute renal rejection model.[13] Thus, the balance between Th17 and Treg might be correlated with successful pregnancy. In addition, IL-17 has a function in recruitment and activation for neutrophils.[14] As an inflammation is involved in inducing abortion, Th17 may play a role in the pathogenesis of abortion. In this study, we examined Th17 cells in the decidua of spontaneous abortion cases in humans.

Materials and methods

Tissue Collection

All samples for this study were approved by the University of Toyama Ethics Committee, and informed consent was obtained from all patients. Ten specimens from cases of elective termination of pregnancy (maternal age median: 28 years, range: 24–37 years; gestational age median: 8 weeks, range: 6–10 weeks) were obtained. These specimens were treated as normal pregnant subjects. Gestational age was calculated from the last menstrual period and confirmed by ultrasound measurements of crown-rump length. Seventeen samples from first-trimester spontaneous abortion cases (maternal age median: 30 years, range: 17–38 years; gestational age median: 7 weeks, range: 4–9 weeks) were collected. Anembryonic pregnancies or fetal death was confirmed by ultrasonography. These samples were divided into two groups: missed abortion and inevitable abortion. A missed abortion was defined as a nonviable pregnancy without vaginal bleeding, uterine cramping, or cervical dilatation. An inevitable abortion was defined when there was active vaginal bleeding and an open external cervical os. All samples were collected by vaginal curettage. In inevitable abortion, curettage was carried out within 12 hr of diagnosis. Both groups were subjected to the same exclusionary criteria: women receiving any medication or with autoimmune diseases or other systemic or local diseases were excluded. Clinical details were recorded for each woman (Table I). The tissue samples were fixed in formalin and embedded in paraffin blocks for histological examination and immunohistochemical staining.

Table I Clinical data from patients with control, missed abortion and inevitable abortion

		Spontaneous abortion	
	Normal control	Missed abortion	Inevitable abortion
n	10	7	10
Age (years)	28 (24–37)	31 (26–38)	29 (17–37)
Gravidity	1 (0–4)	1 (0–3)	0 (0–7)
Parity	0 (0–3)	0 (0–2)	0 (0–1)
No. of Sp-ab* (*n*)	2	2	3
Gestational age (weeks)	8 (6–10)	8 (5–9)	7 (4–9)

Data are expressed as median (range).
*Number of patients with spontaneous abortion in past history, excluding the abortion cases discussed in this study.

Immunohistochemistry

Five-micron sections from formalin-fixed, paraffin-embedded human chorionic tissues were deparaffinized in xylene and rehydrated in graded alcohols, before being subjected to antigen retrieval by immersion in 1% sodium citraconic acid in aqueous solution (Nissin EM, Tokyo, Japan) and irradiated with standard microwave equipment (maximum 500 W; Sharp, Tokyo, Japan) for 15 min. After the tissue samples had been cooled down to 37°C at room temperature, endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 5 min. After non-specific staining had been prevented by soaking the sections in 10% rabbit serum, they were incubated with anti-human CD3 mouse mAb (1:100; Novocastra, Newcastle, UK) or goat polyclonal anti-human IL-17 (1:100; R&D, Minneapolis, MN, USA), before being intermittently irradiated (4 s irradiation, 3 s rest) using specialized microwave equipment (MI33; Azumaya, Tokyo, Japan) for 15 min to improve the immunostaining and then incubated for 30 min at room temperature.[15,16] Further processing of the sections for detection was performed using the dextran-polymer method (Dako, Denmark) and diaminobenzidine (DAB; Sigma, UK). After being washed, the sections were counterstained with Mayer's hematoxylin, washed in water, and successively immersed in graded ethanol solutions and xylene before coverslipping. In the control sections, the primary antibody was replaced by control non-immune goat IgG (Vector Laboratories, CA, USA). Specific IL-17 staining was confirmed by recombinant IL-17 treatment. All samples were processed

under the same conditions. When counting the number of IL-17-positive cells in the IL-17 staining tissues samples, at least three high-power fields were chosen randomly on each sample. Additionally, the number of neutrophils, which have a lobulated nucleus, was counted in the same fields as used for the IL-17 counting in the hematoxylin–eosin-stained samples.

Flow Cytometry

Decidual tissues from missed abortion cases were used for flow cytometry because the samples from the inevitable abortion cases had degenerated. Decidual mononuclear cells (leukocytes) were purified by the Ficoll–Hypaque method after homogenization and filtration through a 32-μm nylon mesh. Decidual tissues were not enzymatically digested so as to prevent the possibility that enzymatic treatment would affect the fluorescence intensity of surface antigens. Decidual mononuclear leukocytes were stimulated with phorbol myristate acetate (PMA, 10 ng/mL; Sigma Chemical Co., Deisenhofen, Germany) and 1 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. These cells were stained for 20 min at room temperature with FITC-conjugated mAb to CD4 or CD8 (BD PharmingenTM, San Diego, CA, USA). The cells were then washed and fixed in 4% formaldehyde/PBS for 5 min at room temperature, before being treated with permeabilizing solution buffer (BD Bioscience, San Jose, 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 the CellQuest software (BD Bioscience). We counted 50,000 cells in each sample. A gate was set on the lymphocytes using characteristic forward scatter (FSC) and side scatter (SCC) parameters. The analyses of CD4 and CD8 staining were performed using the obtained decidual mononuclear cells. 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 the range. P-values < 0.05 were considered significant. The frequency of IL-17-positive cells was analyzed with Mann–Whitney U-test. Spearman rank correlation coefficient was used to determine associations between the numbers of IL-17-positive cells and neutrophils.

Results

Accumulation of IL-17-Positive Cells in Decidua from Spontaneous Abortions

We first examined IL-17 expression in abortive samples obtained from spontaneous abortion cases by immunohistochemistry. Numerous IL-17 antibody-reacted cells were detected in the spontaneous abortive decidual samples (Fig. 1b). Almost all the cells had a round shape and were located in the stroma or blood vessels, suggesting that they were leukocytes (Fig. 1b, arrowheads and arrows). Subsequently, when CD3 staining was performed with serial sections of spontaneous abortive samples, many $CD3^+$ T cells, which had infiltrated into the stroma, were detected in the same area, suggesting that the $IL\text{-}17^+$ cells were T cells (Fig. 1a). On the other hand, $IL\text{-}17^+$ cells were rare in the decidua of the elective termination samples, in which T cells were recognized. These results suggested that the number of $IL\text{-}17^+$ cells is increased in spontaneous abortion, which causes T-cell infiltration.

IL-17-Producing Cells in Decidual $CD4^+$ T Cells and $CD8^+$ T Cells

We next examined whether T cells produce IL-17 in decidual lymphocytes by flow cytometry. The main population of IL-17-producing cells was $CD4^+$ T cells, on the other hand, very few $CD8^+$ T cells produced IL-17 in the spontaneous abortion cases (Fig. 2), suggesting that the decidual $IL\text{-}17^+$ cells were $CD4^+$ Th17 cells. The main population of decidual lymphocytes was $CD56^{bright}$ NK cells, which belong to $CD4^-$ and $CD8^-$ cell population. $IL\text{-}17^+$ cells were very rare in the $CD4^-$ cell population, suggesting that $CD56^{bright}$ NK cells do not produce IL-17.

Increase in the Number of Decidual IL-17-Positive Cells in Inevitable Abortion Cases

We next focused on the localization of $IL\text{-}17^+$ cells in spontaneous abortion cases. $IL\text{-}17^+$ cells were distributed over the entire region of the decidua, the

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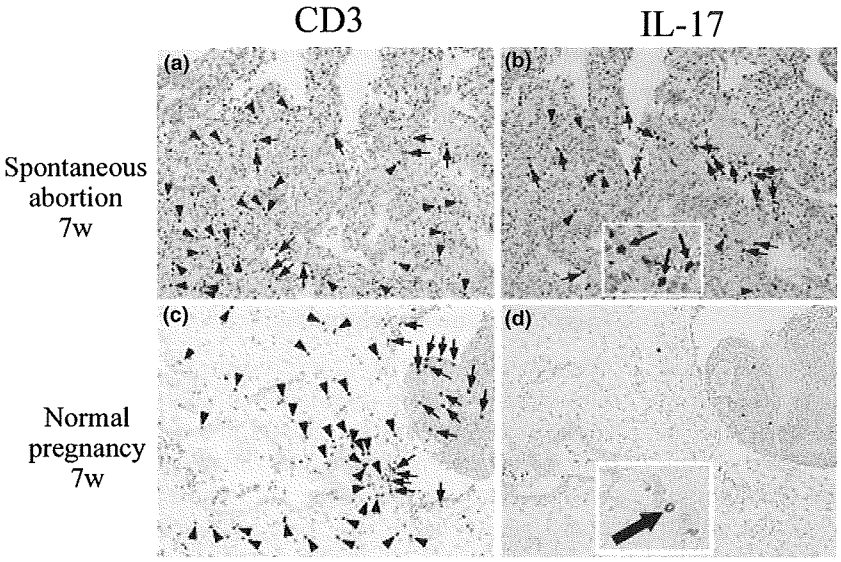

Fig. 1 IL-17 expression in the decidua of spontaneous abortion cases: Serial paraffin sections of the decidua were stained with anti-CD3 (left panels) and anti-IL-17 (right panels) in spontaneous abortion (upper panels) or normal pregnancy (lower panels) cases of 7-week gestation. The expression of IL-17 was detected in the stroma (arrowheads) and blood vessels (arrows) of spontaneous abortion cases, but not those of normal pregnant subjects. The small region outlined by the white line shows IL-17-positive cells (arrows) and has been highly magnified in panels (b) and (d).

LOW RESOLUTION FIG

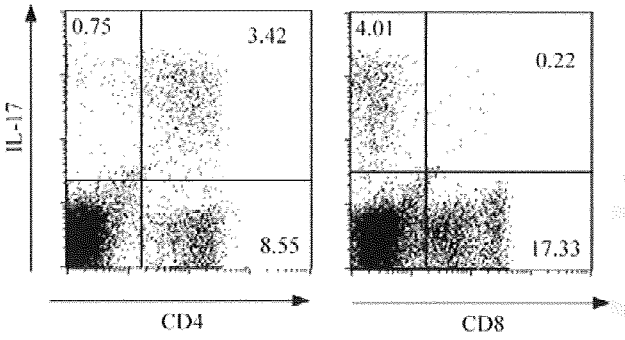

Fig. 2 IL-17 expression in decidual lymphocytes: The intensity of IL-17 staining is shown on the *y*-axis, while the intensity of $CD4^+$ (left panel) or $CD8^+$ (middle panels) staining is plotted on the *x*-axis. The numbers represent the percentages of dots in each gated area.

cell column in the decidua basalis, as well as the decidua parietalis (Fig. 3a–c). Around the cell column, $IL\text{-}17^+$ cells were detected not only in the blood vessels (Fig. 3b, arrows) but also in the stroma (Fig. 3b, arrowheads), suggesting that $IL\text{-}17^+$ cells might infiltrate from blood vessels and into the stroma. However, these cells were absent in the villous trophoblastic layer (Fig. 3d). Additionally, we found differences in the number of $IL\text{-}17^+$ cells in the decidua among spontaneous abortion samples. Therefore, we divided the spontaneous abortion samples into two groups: inevitable abortion and missed abortion according to the presence or absence of symptoms, such as genital bleeding and lower abdominal pain. Subsequently, we compared the number of $IL\text{-}17^+$ cells among the three groups: normal pregnancy, missed abortion, and inevitable abortion. The median values and the ranges of $IL\text{-}17^+$ cell numbers were 0 (0–21), 0 (0–25), and 7 (0–34) in normal pregnant women, missed abortion cases, and inevitable abortion cases, respectively (Fig. 4). Interestingly, the number of $IL\text{-}17^+$ cells in the inevitable abortion cases was significantly higher than that in the normal pregnancy cases (Fig. 4, $P < 0.05$). These data showed that the number of $IL\text{-}17^+$ cells was significantly increased in the inevitable abortion cases but was not changed in the missed abortion cases.

Coexistence of the IL-17-Positive Cells and the Neutrophils in the Inevitable Abortion Cases

As IL-17 is a pro-inflammatory cytokine that plays an important role in neutrophil infiltration, we next examined the correlation between the number of $IL\text{-}17^+$ cells and the number of neutrophils in the inevitable abortion cases. After counting the numbers of $IL\text{-}17^+$ cells and neutrophils around the $IL\text{-}17^+$ cells in high-power fields, the correlation between the numbers of $IL\text{-}17^+$ cells and neutrophils was analyzed in the spontaneous abortion cases. The index of correlation was 0.89, and a significant positive correlation was observed between the number of $IL\text{-}17^+$ cells and the number of neutrophils in the spontaneous abortion cases (Fig. 5, $P < 0.0001$). On the other hand, few neutrophils were detected in the

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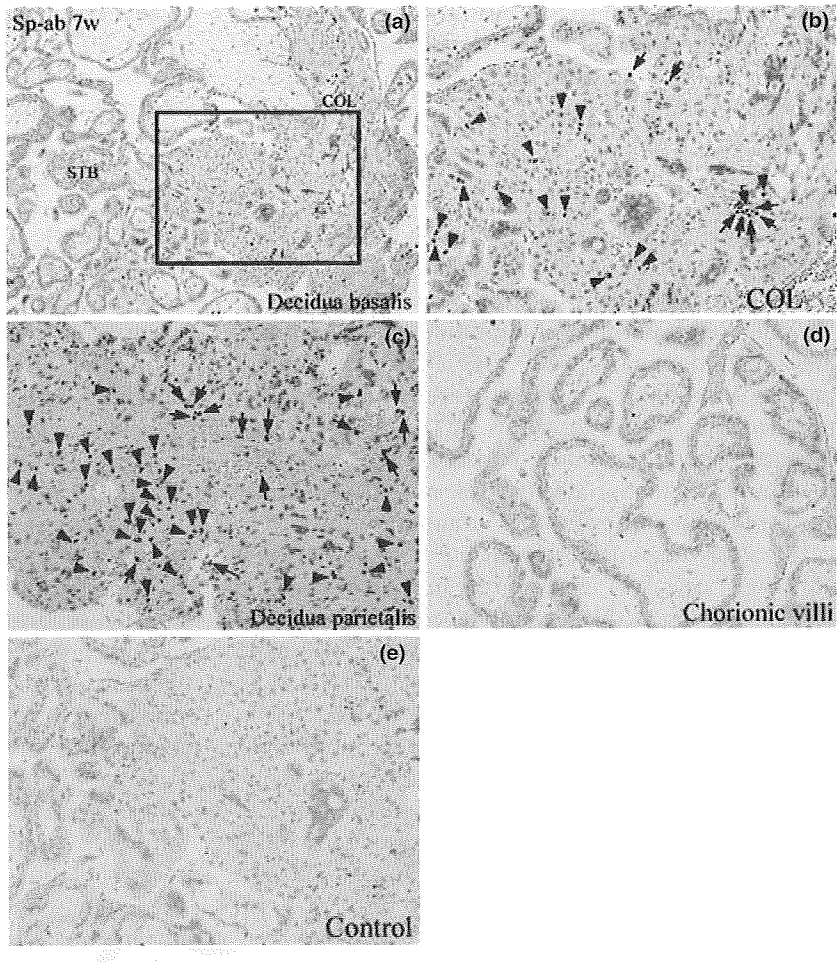

Fig. 3 Distribution of IL-17$^+$ cells in inevitable abortion cases of 7-week gestation: Our immunohistochemical study showed the IL-17 expression in the decidua basalis (a), decidua parietalis (c), and villous (d) of inevitable abortion cases. Panel (b) shows the region outlined by a black line in panel (a). In the control sections, the primary antibody was replaced by control non-immune goat IgG (e). The expression of IL-17 was detected in the decidual stroma (arrowheads) and blood vessels (arrows), but not in chorionic villi. COL, cell column. The cell column was localized on the left side of panel (b).

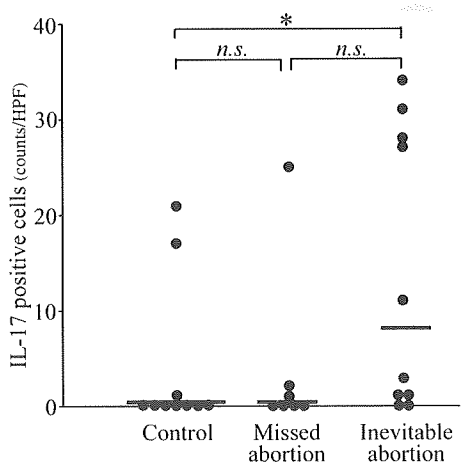

Fig. 4 Comparison of the numbers of IL-17$^+$ cells in deciduas: The numbers of IL-17$^+$ cells in deciduas from normal pregnancy, missed abortion, and inevitable abortion cases. The bars indicate the median values. *$P < 0.05$.

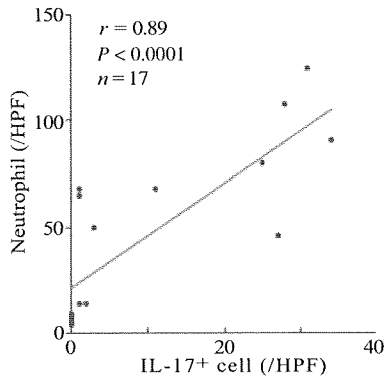

Fig. 5 Correlation between the numbers of IL-17$^+$ cells and neutrophils: A scatter graph was constructed between the numbers of IL-17$^+$ cells (*X*-axis) and neutrophils (*Y*-axis) in spontaneous abortion cases. The coefficient of correlation (*r*) is shown on the upper side of the graph. The line indicates the regression line.

normal pregnant subjects. These results showed that the coexistence of IL-17-positive cells and neutrophils was detected in the late stage of spontaneous abortion.

Discussion

The etiology of spontaneous abortion varies, including chromosomal aberrations, anatomic anomalies, endocrine disorders, infections, reproductive antiphospholipid syndrome, and immunologic abnormalities.[17] Predominant Th1 type immunity might induce abortion;[2,3] however, recent studies have revealed the specific functions of Th17 cells beyond their previously described effects on Th1 and Th2 immunity, including specific roles in host defense against certain pathogens and in autoimmunity.[5–9]

This study demonstrated that the number of decidual IL-17$^+$ cells was increased in inevitable abortion cases involving active genital bleeding, but not in missed abortion cases without symptoms. The main population of these IL-17$^+$ cells was CD4$^+$ T cells, suggesting that decidual IL-17$^+$ cells are Th17 cells. Interestingly, Th17 cells coexisted with neutrophils in the inevitable abortion patients. Recent data that IL-17 plays important roles in the induction of neutrophil-mediated protective immune responses against extracellular bacteria and fungal pathogens support our findings.[18] Th17 cells also play an important role in the induction of inflammation.[19] In the obstetrics and gynecologic field, it has been reported that IL-17 stimulates IL-8 production in endometriotic stromal cells[20] and amniotic mesenchymal cells in chorioamnionitis.[16] IL-17 also enhances (TNF)-α-induced IL-8 secretion by amniotic mesenchymal cells.[16] Thus, (TNF)-α and IL-17 might cooperatively augment IL-8 secretion, resulting in neutrophil accumulation at the decidua in inevitable abortion. In this study, the number of IL-17$^+$ cells did not increase in the missed abortion cases without clinical symptoms. Our recent study showed that the number of circulating Th17 cells did not change during pregnancy and that the proportion of Th17 cells in the decidua was significantly higher than that in the peripheral blood.[21] These findings suggest that IL-17 plays a role in the maintenance of pregnancy during the early pregnant period. Indeed, it has been reported that IL-17 augments extravillous trophoblast invasion.[22,23] However, in the late stage of spontaneous abortion, excessive IL-17 expression may induce neutrophil accumulation, resulting in tissue degeneration or the onset of clinical symptoms. Thus, IL-17 expression level may be involved in a successful pregnancy.

Three major populations in the decidual leukocytes have been identified: uterine natural killer cells, macrophages, and T lymphocytes. Our previous report showed that the number of granulysin$^+$ decidual NK cells was increased in the decidua basalis in spontaneous abortion cases and that these NK cells induced apoptosis in extravillous trophoblasts.[24] This study showed that IL-17$^+$ cells were distributed over the entire region of the decidua, decidua basalis, and the decidua parietalis, in the inevitable abortion cases, but IL-17$^+$ cells did not increase in the missed abortion cases, suggesting that IL-17 expression is not the cause of such abortions but rather is the result of inflammation caused by tissue degeneration or infection. In regard to the IL-17 expression in decidual leukocytes, we have already reported that decidual CD56^{bright} NK cells did not produce IL-17.[16] IL-17 expression was identified in not only CD4$^+$ T cells but also monocytes;[25] however, our previous study showed no IL-17 expression in CD14$^+$ cells in decidual leukocytes.[16] And the population of IL-17$^+$ cells in monocyte area detected by forward and SCCs in flow cytometry was only 0.14%.[16] There are two types of macrophages in the decidua. CD14$^-$ CD68$^+$ macrophages predominate in decidua, while CD14$^+$ CD68$^-$ macrophages are found in superficial myometrium, and the biological significance of these two macrophage populations is unclear.[26] CD4 is also expressed on macrophage, but the staining intensity is rather weaker than that on T cells. In this study, IL-17 expression was detected in CD4^{bright} cell population, suggesting that the main population of IL-17-producing cells is CD4$^+$ T cells, and IL-17-producing CD14$^-$ macrophage is very few (0.14%) in the decidua.

In conclusion, decidual IL-17$^+$ cells were increased in the inevitable abortion cases involving active genital bleeding, but not in missed abortion cases without clinical symptoms. Furthermore, the number of IL-17$^+$ cells was significantly positively correlated with the number of neutrophils, suggesting that IL-17$^+$ cells might be involved in the inflammation in the late stage of abortion, but not in the early stage of abortion. Further studies are needed for understanding the role of Th17 cells in unexplained cases of recurrent pregnancy loss with normal fetal chromosomal content.

Acknowledgement

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Immune Surveillance during Pregnancy

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Introduction

In 1953 Peter Medawar proposed that human pregnancy represents a semiallograft to the maternal host, therefore the process of implantation should include mechanisms preventing allograft rejection. Since then, many researchers have tried to resolve this mechanism. The aim of this study is to discuss immune surveillance during pregnancy.

Role of dendritic cells (DCs) in implantation

Among antigen presenting cells, the most potent inducers of primary immune responses are dendritic cells (DCs). DCs initiate and coordinate the innate and adaptive immune responses. DCs increase their numbers in the uterus during the peri-implantation period (1). Krey et al. firstly reported that DC depletion dramatically impaired implantation using a transgenic mouse system (DTRtg) that allows transient depletion of $CD11c^+$ cells in vivo through administration of diphtheria toxin (2). The same data have also been reported in other studies (3). These findings suggest that fetal-antigen recognition by DCs is necessary for implantation. But Plaks et al. reported that depletion of DCs also causes embryo resorption in syngeneic and T cell-deficient pregnancy, suggesting that DCs appear to govern uterine receptivity by regulating tissue remodeling and angiogenesis, independent of the immunological tolerance. They showed DCs produced sFlt 1 and TGF-β1 that promote coordinated blood vessel maturation. These findings suggest that uterine DCs are crucial for decidual formation during embryo implantation in mice. Furthermore, the maturity of uterine natural killer (uNK) cells was impaired at DC knockout implantation sites (2), and DCKO mice exhibited substantial anomalies in placental development (2). Human decidua contains potent immunostimulatory $CD83^+$ DCs and these DCs contact uNK cells (4). DC-$SIGN^+$-DCs are absent in the non-pregnant uterus in Rhesus Macaque, but uterine DC-$SIGN^+$ DCs increased in number within 1 week of implantation, and these cells are found only adjacent to the implantation site (5). These findings suggest that DCs play an important role in immunology and reproduction, especially in implantation.

Role of $CD4^+$ $CD25^+$ regulatory T cells in allogeneic pregnancy

$CD4^+$ $CD25^+$ regulatory T (Treg) cells play central roles for immune regulation (6). They express high levels of CD25 (IL-2Rα), as well as the cytotoxic T-lymphocyte antigen 4 (CTLA-4) and the transcription factor, Foxp3. Treg cells have potent regulatory properties in both the induction and maintenance phase of *in vivo* tolerance in mice and humans.

Aluvihare et al. reported interesting findings that suggest Treg cells might regulate maternal tolerance to the fetus (7). BALB/c derived–total lymphocytes were injected into T-cell deficient BALB/c nu/nu female mice (Fig. 1). These mice were mated to C57BL/6 male mice, resulting in normal pregnancy. When BALB/c $CD25^+$-cells

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with deleted lymphocytes ($CD25^-$ lymphocytes) were injected into BALB/c nu/nu mice, they did not have $CD4^+CD25^+$ Treg cells (Fig. 1). These Treg cell deficient BALB/c mice were mated with allogeneic C57BL/6 male mice, and abortion occurred (Fig. 1). However, when these Treg cell deficient BALB/c mice were mated to syngeneic BALB/c male mice, they showed normal pregnancy, suggesting that allogeneic fetuses were rejected when $CD4^+CD25^+$ Treg cells were absent. Zenclussen et al. reported that anti-CD25 monoclonal antibody treatment on day 2.5 of gestation induced implantation failure in allogeneic pregnancy, but not in syngeneic pregnancy (8). These data suggest that $CD25^+$ cells play an important role for maintenance of allogeneic pregnancy. $CD4^+$ $CD25^+$ Treg cells might mediate maternal tolerance, but $DX5^+CD25^+$ NKreg cells might also play some roles in successful pregnancy (see Chapter b!). We should clarify which cells are important for maintenance of pregnancy. In humans, our group firstly reported that $CD4^+CD25^{bright}$ Treg cells dramatically increased in early pregnancy decidua. And these increased decidual Treg cell ratios were decreased in spontaneous abortion or habitual abortion (9). These findings suggest that increased Treg cells at the fetomaternal interface might play an important role for the maintenance of allogeneic pregnancy.

As a mechanism of immunoregulation, immunostimulation of Treg cells is important. As a first step, Treg cells recognize some antigens via T cell receptors/CD3 complex. At the same time, CD28 on Treg cells bind B7 complex on DCs. T cell receptors and CD28-mediated costimulation are required for Treg cells to exert suppression. These activated Treg cells express CTLA-4 on their surface, and can suppress both $CD4^+$ T cells and $CD8^+$ T cells by cell-to-cell interaction. Therefore, surface CTLA-4 expression on Treg cells is a marker for activated and functional Treg cells. Interestingly, surface CTLA-4 expression on Treg cells increases in decidual Tregs but not in peripheral blood Treg cells of early pregnancy subjects (9). This increased surface CTLA-4 expression decreases in miscarriage cases (9), suggesting that functional Treg cells might induce alloantigen-specific tolerance, resulting in maintenance of pregnancy. Jasper et al. reported that primary unexplained infertility is associated with reduced expression of the Treg cell transcription factor Foxp3 in endometrial tissue (10). Recent data demonstrate that expansion of the Treg cells during pregnancy induces tolerance to paternal alloantigens in mice (11). Expansion of Treg cells in para-aortic lymph modes draining the uterus is observed on day 3.5. This increase in Treg cells is

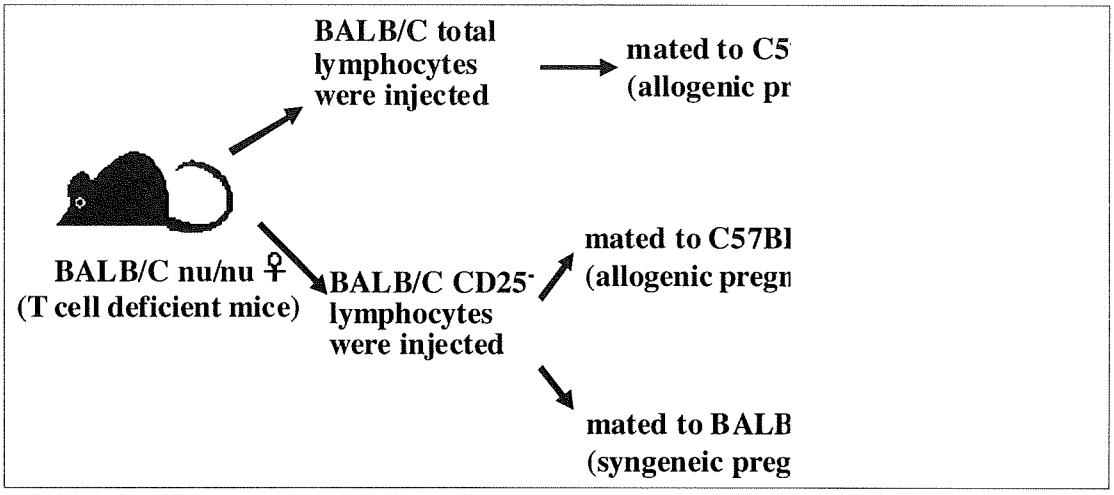

Fig. 1: Regulatory T cells mediate maternal tolerance to the fetus.

abrogated when males are vasectomized or seminal vesicles are excised (11). These findings suggest seminal fluid plays an important role for induction of tolerance to paternal alloantigens in mice.

Role of regulatory NK cells during pregnancy

NK cells are one of the key cell types involved in allograft rejection. However, in certain transplant models, NK cells also express potent immunoregulatory properties such as tolerance induction.

We have reported that leukemic peripheral blood $DX5^+$ $CD25^+$ $Thy1.2^{++}$ $c\text{-}kit^+$ NK cells have immunoregulation ability such as inhibition of allo-T cell stimulatory activity of DCs and autotumor specific CTL induction in mice (12). When the myelomonocytic leukemia cell line, WEH1 3B, is injected into BALB/c mice, $CD3^-$ $DX5^+$ NK cells are rapidly increased in the peripheral blood.

These phenotypes are quite different from those of conventional NK cells (Table 1). These NK cells express CD25, Thy-1.2 and c-kit. Thy 1.2 and c-kit are markers for progenitor cells, suggesting that these leukemic NK cells are an immature type of NK cells. Leukemic NK cells downregulate the expression of MHC class a! antigen on DCs mediated by TGF-â production. These NK cells suppress the allo-T cell stimulatory activity of DCs. These NK cells inhibit generation of autotumor-specific CTL, suggesting that these NK cells are regulatory NK cells. Very interestingly, these $CD25^+$ $Thy1^{high}$ $c\text{-}kit^{high}$ NK cells accumulate in the pregnant uterus of BALB/c mice. And most of the decidual lymphocytes in NOD/SCID mice are $CD3^-$, $Ly49^-$, $CD25^+$, $Thy1^{high}$, $c\text{-}kit^{high}$ cells. Most of the uterine NK cells produce IL-10 and 10-20% of the uterine NK cells produce TGF-βin SCID mice.

In BALB/c mice, 30% of uterine lymphocytes are NKreg cells, but in NOD/SCID mice, this

Table 1. The phenotypes of regulatory NK cells and conventional NK cells in mice.

	Regulatory NK cells	Conventional NK cells
Surface marker		
CD94	negative ~ low	high and low
Ly49 C/F	low	high and low
acialo GMI	high	high
CD25	high	negative-low
CD122	high	high
Thy-1, 2	very high	medium
c-kit	medium ~ high	negative ~ low
Cytokine production		
IFN-0	low	high
IL-4	low	low
TGF-0	medium	low
IL-10	high	low
Ctytoxic activity	low	high
TLR3 stimulation	low activation	high activation
TLR4 stimulation	low activation	high activation

population is 85-90%. Poly (1:C) treatment or LPS treatment on gestational day 8.5 and 9.5 induce abortion. But these treatments do not induce abortion in NOD/SCID mice. NK activity in spleen, peripheral blood and the uterus are elevated by the treatment of Poly (1:C) or LPS in BALB/c mice. But in NOD/SCID mice, these treatments do not augment the NK cell activity. These findings suggest that NKreg cells might regulate inflammation, resulting in maintenance of pregnancy (Table I).

Our group already reported that CD25 and c-kit are expressed on decidual $CD56^{bright}$ NK cells in humans (14, 15). And we also reported that IL-10 producing NK cells increase in peripheral blood, and TGF-β producing NK cells increase in decidua of early pregnancy subjects (16). These findings suggest NKreg cells are also present in human pregnancy, and they may play important roles for maintenance of pregnancy.

Conclusion

Many papers support the idea that immune cells play important roles for successful implantation and pregnancy. These data may assist in resolving the limited implantation success of embryos transferred following IVF-ET.

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BRIEF COMMUNICATION

Japanese single women have limited knowledge of age-related reproductive time limits

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In 2007, the total fertility rate in Japan decreased to 1.34, compared with 2.10 in the USA. This decline in fertility is partly due to increased age at marriage and delay in childbearing. Increased maternal age is associated with an increased risk of infertility, miscarriage, and poor pregnancy outcome [1]. A 1986 study found that the percentage of women who did not use contraception and remained childless increased steadily according to age at marriage (6% among 20–24-year-old women compared with 64% among 40–44-year-old women) [2]. The miscarriage rate rose from 14% among patients younger than 35 years of age to 40% among those older than 40 years of age [3]. The average childbearing age in Japan has increased over the past 3 decades as more women have postponed marriage to pursue higher education and careers.

Between June 2007 and March 2008, a 2-page anonymous survey was distributed at Nagoya City University, Nagoya Women's University, and a Women's Health Week in Nagoya (sponsored by the Japanese Society for Obstetrics and Gynecology). In total, 249 single women completed the 15-item survey, which contained questions addressing attitudes toward marriage, occupation, and childbirth, in addition to knowledge about infertility, miscarriage, and age-related reproductive time limits. The Research Ethics Committee at Nagoya City University Medical School approved the study.

The mean age of participants was 25.2 ± 6.8 years. In total, 95.5% of participants expressed a desire to marry in the future, with 96.8% wanting to continue work after marriage; however, 25.7% wanted to stop working after childbirth. Overall, 91.0% of women wanted to have children, and 85.5% thought that childbirth would be important in their life. The issues of marriage, career, and childbirth had been seriously considered by 46.2%, 67.9%, and 40.6% of single women, respectively. The mean age of women considering marriage and childbirth was higher than that of women not considering these events and of women who reported that they were influenced by parental values or common social practice (Table 1).

Nearly all women (98.8%) were familiar with the term "infertility," although only 44.2% and 17.3% chose accurate rates of infertility and miscarriage, respectively. Only 10.7% chose correct answers for all aspects of reproduction. Surprisingly, 36.4% estimated their own age limit for natural pregnancy to be between 45 and 60 years. Significantly more women considering marriage and childbirth chose correct responses about the rate of infertility and about reproduction. Women considering a career chose rates of infertility and miscarriage more accurately than did women not considering this option.

Regarding questions about the source of their knowledge of the term "infertility," 85.9% of women had learned the term from the media, compared with 20.7% who had learned it from school teachers. There was no difference in overall knowledge between women who obtained their information from school teachers and those who obtained it from other sources. Women considering marriage and those with accurate knowledge of infertility rates and causes tended to want children later. Participants with an accurate knowledge of infertility causes incorrectly chose a significantly older fertility time limit. Older participant age was significantly associated with knowledge of infertility rates; however, older participants wanted children later and believed reproductive time limits to be over 40 years of age.

In the present study, there were deficits in the participants' knowledge, despite the majority reportedly knowing the term "infertility." The terms "birth control," "contraception," "induced abortion," and "sexually transmitted infections" can be found in secondary-school health and physical education, biology, and domestic science textbooks, but "infertility" is seldom seen—indicating that there is no substantial public education in Japan about this condition. Women who reported that they had gained their knowledge of reproductive health from school teachers were unaware of accurate infertility rates. This raises concern that school teachers have limited knowledge on the subject, and indicates a need for infertility content to be added to the secondary-school curriculum.

The findings from the present study imply that, without knowing their reproductive limits, many Japanese women may lose their capacity for conception; thus, increased efforts are needed to educate

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Table 1
Association between knowledge about reproductive health issues and personal aspirations[a].

	Age	Considering marriage	Considering career	Considering childbirth	School teachers listed as source of knowledge	Expected age at childbirth	Knowledge of reproductive time limit	Over 40 years of age
Age		<0.01 Yes 26.8 ± 7.4 No 23.7 ± 5.9	>0.05 Yes 25.7 ± 7.0 No 24.1 ± 6.4	<0.01 Yes 26.4 ± 7.4 No 24.4 ± 6.3				
Knowledge about infertility rate	0.01	<0.01	0.07	0.03	>0.05	0.08 (late)	>0.05	>0.05
Knowledge about male:female causes	>0.05	>0.05	0.05	>0.05	>0.05	0.07 (late)	0.01 (late)	>0.05
Knowledge about miscarriage rate	>0.05	>0.05	0.04	>0.05	>0.05	>0.05	>0.05	>0.05
Knowledge about all aspects of reproduction	0.07	0.04	>0.05	0.03	>0.05	0.09 (late)	>0.05	>0.05
Expected age at childbirth	<0.01 (late)	0.07 (late)	>0.05	>0.05	0.10 (early)			
Knowledge of reproductive time limit		>0.05	>0.05	>0.05	>0.05			
Over 40 years of age	0.06 Yes 25.4 ± 6.9 No 24.8 ± 6.5	>0.05	>0.05	>0.05	>0.05			

[a] Values are given as mean ± SD or *P* values.
[b] $P<0.05$ was considered statistically significant.

Japanese women about the influence of age on fertility. Societal pressures forcing women to choose between a career and children must change to reverse the very low fertility rate in Japan.

Conflict of interest

The authors have no conflicts of interest.

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Live Birth Rate According to Maternal Age and Previous Number of Recurrent Miscarriages

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Problem
In Japan, marital age and women's age at the first pregnancy are continuing to increase year by year. However, information concerning subsequent live birth rate according to maternal age and number of previous recurrent miscarriages is limited.

Method of study
We studied a total of 1250 unexplained patients suffering two or more consecutive miscarriages. We examined the live birth rate at the first pregnancy and the cumulative success rate for birth of at least one child after examination.

Results
The live birth rate of women in their 40s was 58.1%, which was similar to that of women who were 35–39 years old (58.4%) at the first pregnancy, as found after examination. From logistic regression, women's age and the number of previous miscarriages independently decreased the live birth rate in subsequent pregnancies (p_s) as well as cumulative pregnancies (p_c), as follows:

$$\text{logit}(p_s) = 3.964 - 0.0652 \times (\text{age}) - 0.408 \times (\text{previous number of miscarriages})$$

$$\text{logit}(p_c) = 6.806 - 0.1130 \times (\text{age}) - 0.514 \times (\text{previous number of miscarriages}).$$

Conclusion
The information concerning the live birth rate can be given to each patient before subsequent pregnancy.

Introduction

Marital age and women's age at the first pregnancy are continuing to increase year by year in Japan. Also, total fertility rate has decreased and is now 1.34 in Japan, compared to 2.10 in the USA in 2007.[1] This problem is called as 'Shosika' in Japanese, which means childless society, and is common to many countries. Increasing maternal age is also associated with increase risk of infertility, miscarriage, and poor prognosis in pregnancy, such as pre-eclampsia.[2]

Established causes of recurrent miscarriages are abnormal chromosomes in either partner, particularly translocations, as well as antiphospholipid antibodies (aPLs) and uterine anomalies.[3–5] An abnormal embryonic karyotype is also causative of recurrent miscarriage and has been reported in about 25–50% of aborted conceptions.[6–8] The relatively wide range reported may reflect differences in maternal mean age and previous mean number of miscarriages, as these could conceivably exert an influence.