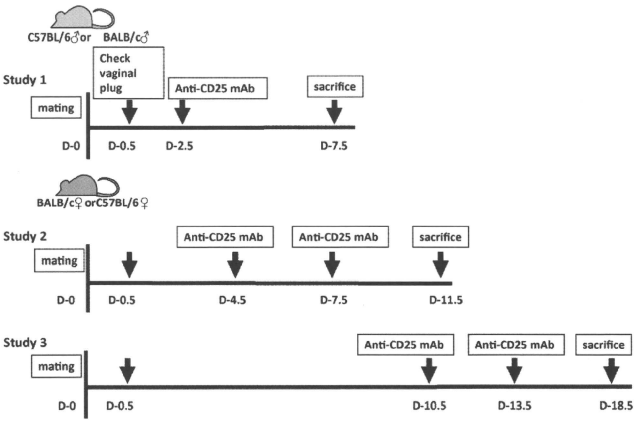


**Fig. 1.** Protocol for administering anti-CD25 monoclonal antibody to pregnant mice. Mice were checked for vaginal plugs on the morning following mating. The day of detection of vaginal plug was designated as day 0.5 post-coitum (pc). Anti-CD25mAb was injected intraperitoneally on day 2.5 pc (study 1), or days 4.5 and 7.5 pc (study 2), or days 10.5 and 13.5 pc (study 3). Pregnant mice were sacrificed on day 7.5 pc (study 1), day 11.5 pc (study 2), or day 18.5 pc (study 3).

USA) was performed on fixed and permeabilized cells using Cytofix/Cytoperm (Cat No: 00-5521, e-Bioscience). After staining, the cells were washed, and analyzed on a FACSCalibur flow cytometer (Becton Dickinson) using Cell Quest software (Becton Dickinson).

### 2.6. Statistical analysis

Statistical differences between groups were determined by Mann–Whitney *U*-test or ANOVA test. $p < 0.05$ was regarded as significant.

## 3. Results

### 3.1. The effect of anti-CD25 mAb treatment on $CD4^+CD25^+$ Treg cell population size and resorption rate

We injected anti-CD25 mAb into pregnant mice on days 4.5 and 7.5 pc to deplete Treg cells in the early pregnancy period. Treg cells were measured as $CD4^+CD25^+$ cells, expressed as a percentage of total $CD4^+$ cells ($\%CD4^+CD25^+/CD4^+$). The $CD4^+CD25^+$ cell population size in the spleen of BALB/c pregnant mice mated with C57BL/6 males and BALB/c pregnant mice mated with BALB/c males were significantly higher than those of non-pregnant BALB/c mice ($p < 0.0001$ and $p < 0.0001$ respectively) (Fig. 2a and c). First, we sought to find the optimal concentration of anti-CD25 mAb to decrease $CD4^+CD25^+$ Treg cells. Administration of 0.1 mg and 0.25 mg anti-CD25 mAb did not reduce the $CD4^+CD25^+$ cell population size in BALB/c pregnant mice mated with C57BL/6 males (Fig. 2a) or C57BL/6 pregnant mice mated with BALB/c males (Fig. 2b). Furthermore, the resorption rate in these groups did not increase (Fig. 2a and b).

On the other hand, administration of 0.375 mg, 0.5 mg and 1 mg of anti-CD25 mAb significantly reduced the $CD4^+CD25^+$ cell population size. The $CD4^+CD25^+$ cell population size decreased to $5.11 \pm 2.51\%$, $2.24 \pm 0.37\%$, and $2.26 \pm 0.95\%$, respectively, from $12.94 \pm 0.91\%$ in BALB/c pregnant mice mated with C57BL/6 males (Fig. 2a). We also confirmed that $CD4^+Foxp3^+$ Treg cells decreased to $3.29 \pm 1.66\%$ from $9.43 \pm 0.40\%$ after administration of 0.5 mg of anti-CD25 mAb. In these groups, the resorption rate increased to $36.4 \pm 11.6\%$, $56.9 \pm 27.9\%$ and $53.4 \pm 3.0\%$ in BALB/c pregnant mice mated with C57BL/6 males, and $36.9 \pm 13.9\%$, $55.6 \pm 29.3\%$ and $53.3 \pm 7.4\%$ in C57BL/6 pregnant mice mated with BALB/c males when administration of 0.375 mg, 0.5 mg and 1 mg of anti-CD25mAb was performed (Fig. 2a and b).

These findings suggested that when the number of Treg cells was reduced to below 50%, the resorption rate increased. Hematoxylin and eosin staining of resorbing fetuses showed hemorrhage at the feto–maternal interface as well as lymphocyte infiltration (Fig. 2d). Treatment with anti-CD25 mAb (0.5 mg) significantly reduced the $CD4^+CD25^+$ cell population size in both allogeneic (Fig. 2a and b) and syngeneic pregnancy (Fig. 2c). Depletion of $CD4^+CD25^+$ Treg cells induced spontaneous abortion in allogeneic pregnancy, however this mAb treatment did not affect the spontaneous resorption rate in syngeneic pregnancy (Fig. 2c).

### 3.2. Treatment with anti-CD25 mAb on day 2.5 pc causes implantation failure in allogeneic pregnancy

To investigate the role of Treg cells in the implantation phase, we injected anti-CD25 into mated mice on day 2.5 pc. Administration of 0.5 mg anti-CD25 mAb

significantly reduced the size of the $CD4^+CD25^+Foxp3^+$ Treg cell population, expressed as a percentage of total $CD4^+$ cells (%$CD4^+CD25^+$ $Foxp3^+/CD4^+$), in spleen and draining lymph nodes in both allogeneic pregnancy and syngeneic pregnancy (Fig. 3). No implantation sites were evident in C57BL/6 pregnant mice mated with Balb/c males after treatment of anti-CD25 mAb, but rat IgG treatment groups showed $11.2 \pm 1.7$ viable fetuses. The number of viable fetuses in the group treated with anti-CD25 mAb was similar to those in the rat IgG treated group in BALB/c pregnant mice mated with Balb/c males (Fig. 3).

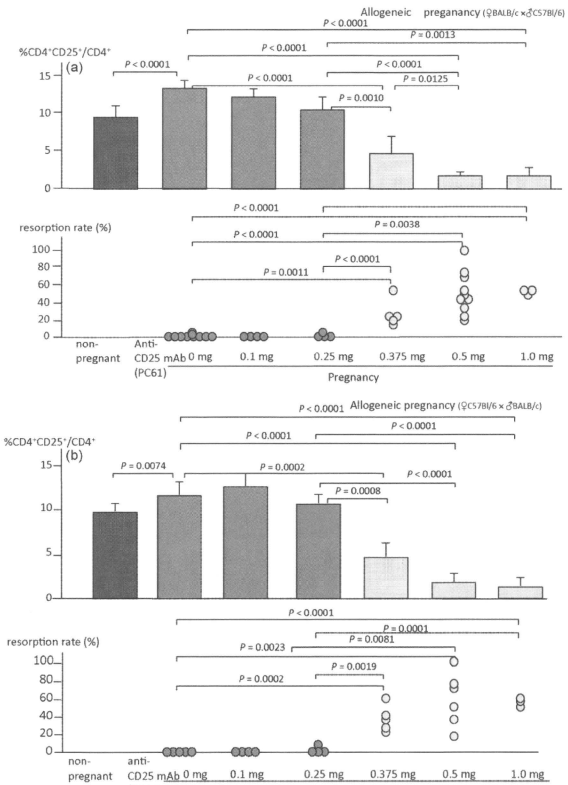


**Fig. 2.** The effect of administering varying amounts of anti-CD25 mAb on days 4.5 and 7.5 pc on the size of the $CD4^+CD25^+$ Treg cell population and the resorption rate (study 2). The size of the $CD4^+CD25^+$ cell population (upper panel) and resorption rate after treatment with the anti-CD25 mAb (lower panel) in BALB/c female mice mated with C57BL/6 males (a) or in C57BL/6 female mice mated with BALB/c males (b). Anti-CD25mAb treatment was performed for each concentration from 0.1 mg to 1.0 mg (a and b). (c) The size of the $CD4^+CD25^+$ cell population (upper panel) and resorption rate (lower panel) after treatment with 0.5 mg of the anti-CD25 monoclonal antibody in syngeneic matings (BALB/c females mated with BALB/c males or C57BL/6 females mated with C57BL/6 males). The size of the $CD4^+CD25^+$ cell population is expressed as a percentage of total $CD4^+$ cells (%$CD4^+CD25^+/CD4^+$). Resorption rate shows the percentage of aborted fetuses in total implantation sites. Error bars represent standard error. (d) Representative photomicrograph of a hematoxylin and eosin stained section of an aborted embryo in an allogeneic pregnancy (C57BL/6 female mated with a BALB/c male) after treatment with anti-CD25 mAb (0.5 mg).

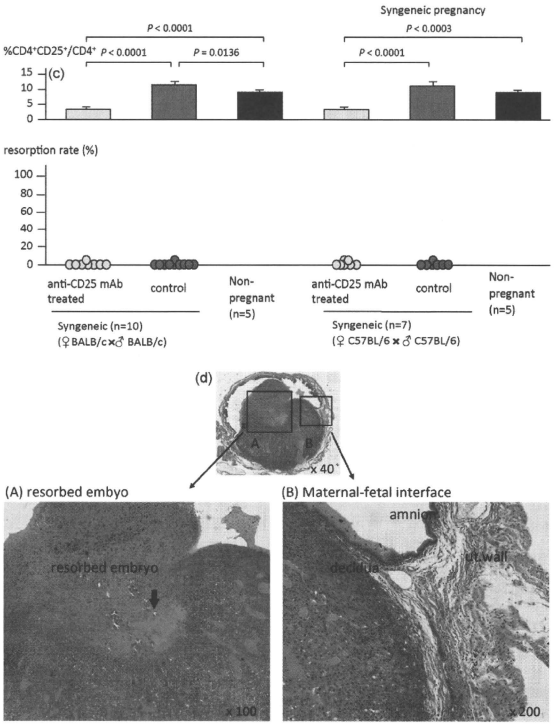


**Fig. 2.** (*Continued*)

### *3.3. Treatment with anti-CD25 mAb on days 10.5 and 13.5 pc did not induce abnormal pregnancy parameters in allogeneic pregnancy*

Administration of 0.5 mg anti-CD25 mAb on days 10.5 and 13.5 pc significantly reduced the $CD4^+CD25^+Foxp3^+$ Treg cell population size in spleen and draining lymph nodes on day 18 in allogeneic pregnancy (Fig. 4). Nevertheless, this treatment did not induce any abnormal pregnancy parameters. The number of viable fetuses in the anti-CD25 mAb treatment group was similar to the rat IgG treated group (Table 1). The weights of the fetus and the placenta were also not different (Table 1).

It has been reported that the Treg cell population size is decreased in preeclampsia (Sasaki et al., 2007; Darmochwal-Kolarz et al., 2007; Santner-Nanan et al., 2009). To investigate the relationship between decrease of Treg cells and preeclampsia, we monitored blood pressure and urinary protein levels in Treg cell depleted mice. Administration of anti-CD25 mAb did not elevate the blood pressure, and did not increase urinary protein levels in allogeneic pregnancy, either in BALB/c pregnant mice mated with C57BL6 males, or in C57BL/6 pregnant mice mated with BALB/c males (Table 1).

## 4. Discussion

The fetus is considered to resemble a semi-allograft in the maternal host, but the healthy fetus survives and maternal rejection is prevented. Therefore, tolerance systems to the fetus are present during pregnancy. In this process, corticotrophin-releasing hormone (CRH) and the Fas ligand (FasL) play some part in T cell clonal deletion. CRH produced by trophoblasts and decidual cells induces FasL expression on trophoblasts, and FasL promotes apoptosis of maternally activated T cells, which express Fas

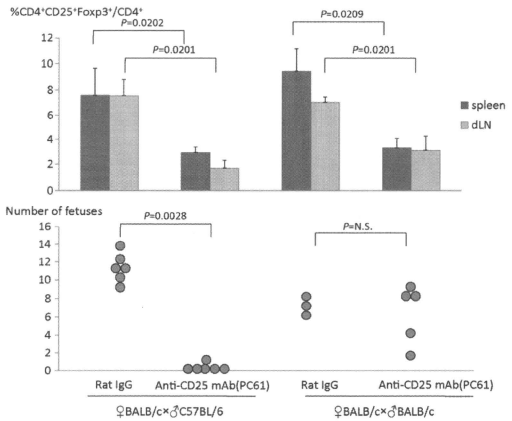


**Fig. 3.** Treatment with anti-CD25 mAb on day 2.5 pc causes implantation failure in allogeneic pregnancy (study 1). The size of the $CD4^+CD25^+Foxp3^+$ Treg cell population in the spleen (dark gray bar) and draining lymph nodes (dLN: light gray bar) (upper panel), and the number of normal viable fetuses per pregnancy (lower panel) after treatment with 0.5 mg of an anti-CD25 mAb on day 2.5 pc in BALB/c females mated C57BL/6 males or BALB/c females mated with BALB/c males. The size of the $CD4^+CD25^+Foxp3^+$ Treg cell population is expressed as a percentage of total $CD4^+$ cells (%$CD4^+CD25^+$ $Foxp3^+/CD4^+$). Error bars represent standard error.

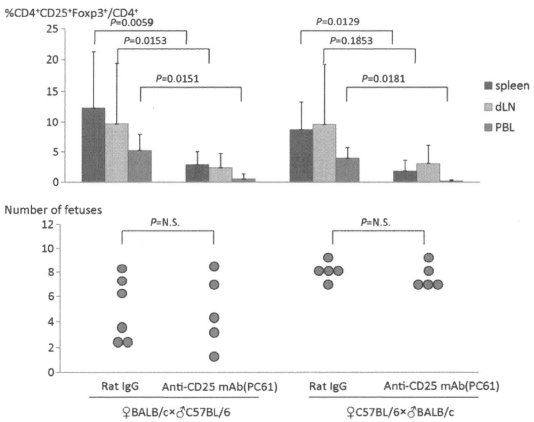


**Fig. 4.** Treatment with anti-CD25 mAb on days 10.5 and 13.5 pc did not induce fetal loss in allogeneic pregnancy (study 3). The size of the $CD4^+CD25^+Foxp3^+$ Treg cell population in spleen (dark gray bar), draining lymph nodes (dLN: light gray bar), and peripheral blood (PBL: middle gray bar) (upper panel), and the number of normal viable fetuses per pregnancy (lower panel) after treatment with 0.5 mg of an anti-CD25 mAb on days 10.5 and 13.5 pc in BALB/c females mated with C57BL/6 males or C57BL/6 females mated with BALB/c males. The size of the $CD4^+CD25^+Foxp3^+$ Treg cell population is expressed as a percentage of total $CD4^+$ cells (%$CD4^+CD25^+$ $Foxp3^+/CD4^+$). Error bars represent standard error.

**Table 1**
The fetal weight, placental weight, blood pressure and proteinuria in study3.

| | BALB/c(♀) × C57BL/6 (♂) | | | C57BL/6 (♀) × BALB/c (♂) | | |
|---|---|---|---|---|---|---|
| | Rat IgG $N=6$ | PC61 $N=5$ | $P$-value | Rat IgG $N=7$ | PC61 $N=6$ | $P$-value |
| Fetal weight (g) | 1.23 ± 0.22 | 1.21 ± 0.15 | 0.8431 | 1.28 ± 0.07 | 1.14 ± 0.22 | 0.2236 |
| Placental weight (g) | 0.20 ± 0.05 | 0.21 ± 0.07 | 0.6886 | 0.13 ± 0.01 | 0.14 ± 0.03 | 0.8419 |
| Blood pressure (mmHg) | | | | | | |
| Day 10.5 | 98.3 ± 8.9 | 110.0 ± 7.0 | 0.0771 | 116.6 ± 5.2 | 109.5 ± 9.1 | 0.2683 |
| Day 13.5 | 107.8 ± 10.3 | 117.0 ± 2.0 | 0.1084 | 118.8 ± 11.6 | 116.5 ± 4.4 | 1.0000 |
| Day 14.5 | 107.5 ± 9.6 | 107.7 ± 6.4 | 0.7237 | 119.3 ± 9.9 | 119.0 ± 9.6 | 0.7188 |
| Day 17.5 | 118.5 ± 6.5 | 120.5 ± 4.9 | 0.6434 | 122.0 ± 6.6 | 120.5 ± 7.4 | 0.7237 |
| Day 18.5 | 114.3 ± 12.3 | 118.5 ± 4.8 | 0.4678 | 119.8 ± 6.2 | 123.5 ± 3.5 | 0.3241 |
| Proteinuria (mg/dl) | | | | | | |
| Day 10.5 | 71.8 ± 44.6 | 100.0 ± 0.0 | 0.2888 | 90.0 ± 26.5 | 80.0 ± 44.7 | 0.7080 |
| Day 13.5 | 76.7 ± 36.1 | 86.0 ± 31.3 | 0.6374 | 95.0 ± 13.2 | 70.8 ± 34.4 | 0.1364 |
| Day 18.5 | 155.0 ± 115.5 | 140.0 ± 89.4 | 1.0000 | 123.6 ± 78.9 | 100.0 ± 0.0 | 1.0000 |

Fetal weight and placental weight were checked on day 18.5. Blood pressure (mmHg) was checked on day 10.5 before anti-CD25 mAb treatment, and checked again over several days. Proteinuria was measured by tape test. Statistical analysis was performed using Mann–Whitney $U$-test.

on their surfaces (Makrigiannakis et al., 2001). Therefore, Fas-mediated death in T cells is one of the mechanisms of tolerance.

As another mechanism, Treg cells play a very important roles for induction and maintenance of tolerance (Sakaguchi, 2004; Wood and Sakaguchi, 2003). The T reg cell population increases in peripheral blood, in decidua (uterus), the iliac lymph node, inguinal lymph node, and in the spleen, and these increased numbers of Treg cells have been shown to suppress alloreactive proliferation *in vitro* (Aluvihare et al., 2004; Sasaki et al., 2004; Heikkinen et al., 2004; Somerset et al., 2004; Zenclussen et al., 2005; Zhu et al., 2005; Robertson et al., 2009). We have reported that $CD4^+CD25^+$ Treg cells increase in early pregnancy decidual tissue in human pregnancy (Sasaki et al., 2004). Aluvihare et al. (2004) reported that when BALB/c derived $CD25^+$ cell-depleted lymphocytes are injected into T cell-deficient BALB/c nu/nu female mice and mated with C57BL/6 males, these mice (with allogeneic pregnancy) undergo abortion, although mating with BALB/c males (syngeneic pregnancy) results in normal pregnancy.

In this study, we performed *in vivo* antibody-mediated $CD25^+$ cell depletion to study the role of Treg cells for the maintenance of allogeneic pregnancy in the implantation period, early pregnancy period and late pregnancy period. When 0.1 mg or 0.25 mg of anti-CD25 mAb were injected into BALB/c females mated with C57BL/6 males in early pregnancy periods (days 4.5 and 7.5 pc), the size of the Treg cell population decreased to 4.9% and 19.5%; however they had successful pregnancy. On the other hand, when 0.375 mg, 0.5 mg or 1.0 mg of anti-CD25 mAb was injected into BALB/c females mated with C57BL/6 males, the reduction of Treg cell population size in the spleen was reduced to 60.5%, 82.7% and 82.6%, respectively, and the resorption rate increased to 36.4 ± 11.6%, 56.9 ± 27.9% and 53.4 ± 3.0%, respectively. It was similar in the case of C57BL/6 females mated with BALB/c males. Interestingly, treatment with anti-CD25 mAb reduced Treg cells in both allogeneic and syngeneic pregnancy, but this mAb treatment did not affect the miscarriage rates in syngeneic BALB/c pregnant mice or C57BL/6 pregnant mice.

Treatment with anti-CD25 mAb in the implantation period (day 2.5 pc) induced implantation failure in allogeneic pregnant mice. Other reports also showed that depletion of Treg cells using 0.2 mg of anti-CD25 mAb on day 2 of pregnancy led to implantation failure (Zenclussen et al., 2005). Administration of anti-CD25 mAb on the day of mating induced expansion of activated $CD8^+$ and $CD4^+$ cell populations in the draining lymph nodes of the uterus and fewer allogeneic fetuses survived to term, whereas no effect was observed in syngeneic pregnancy (Darrasse-Jèze et al., 2006). Recent studies show that the depletion of uterine dendritic cells (DC) induces implantation failure, but depletion of DCs also causes embryo resorption in syngeneic mice (Krey et al., 2008; Plaks et al., 2008). DC appear to govern uterine receptivity in both allogeneic and syngeneic pregnancy. However Treg cells are essential for maintenance of allogeneic pregnancy in the implantation phase and early stage of pregnancy in mice.

Several studies reported an association between Treg cells and implantation failure or recurrent spontaneous miscarriage in humans. We have reported an elevated $CD4^+CD25^{high}$ T cell ratio in peripheral blood and deciduas, and this ratio decreased to a non-pregnancy level in miscarriage cases in humans (Sasaki et al., 2004). Women experiencing repeated miscarriage were shown to have a reduced frequency of Treg cells within peripheral blood, and reduced suppressive capacity, compared with normal fertile women (Arruvito et al., 2007; Yang et al., 2008). Unexplained infertility has also been associated with reduced expression of Foxp3 mRNA in endometrial tissue (Jasper et al., 2006). Other studies show that persistent inhibition of the Toll-like receptor system has a suppressive effect on Treg cells (Pasare and Medzhitov, 2003; Yang et al., 2004). Chronic inflammation and/or decreased Treg cells at the feto–maternal interface might induce implantation failure in IVF-ET or recurrent spontaneous abortion in humans. Recent observations in mice exposed to seminal fluid in the absence of conception support a role for seminal fluid in driving Treg cell activation and proliferation, which promotes tolerance of paternal alloantigens at the time of embryo implantation (Robertson et al., 2009).

Treg cells originally existing in endometrial tissue, as well as Treg cell populations expanded by seminal fluid, may act together to facilitate maternal acceptance at implantation.

In this study, we used administration of anti-CD25 mAb to deplete the $CD4^+CD25^+$ Treg cells. However this mAb treatment also depleted $CD25^+$ NK cells and $CD25^+$ T cells. This is a weak point of this study. We have already reported that $CD25^+DX5^+CD3^+$NK cells have the ability to participate in immunoregulation (Ebata et al., 2006), and these NK cells are increased in the pregnant uterus (Lin et al., 2009). The extent of depletion of $CD25^+DX5^+CD3^+$NK cells was 71% in the draining lymph nodes after administration of anti-CD25 mAb (0.5 mg) in our study. To deplete Treg cells only, Foxp3-DTR mice (Lahl et al., 2007) might be useful. We are planning to study the role of $Foxp3^+$ Treg cells during pregnancy using this animal model.

Some studies have shown a decreased number of Treg cells in preeclampsia. $CD4^+CD25^{high}$ Treg cells were significantly reduced in both the peripheral blood and decidual tissue of preeclampsia patients compared with normal pregnant women (Sasaki et al., 2007; Darmochwal-Kolarz et al., 2007). To investigate the role of Treg cells in the late pregnancy period, we injected anti-CD25 mAb to allogeneic pregnant mice on days 10.5 and 13.5 pc. Anti-CD25 mAb treatment in late pregnancy phase reduced the Treg cells in a similar manner to treatment on day 2.5 pc or days 7.5 and 10.5 pc, but interestingly this treatment did not induce preeclampsia symptoms such as hypertension, proteinuria, or intrauterine growth restriction. We could not demonstrate a requirement for Treg cells in the late pregnancy phase in allogeneic pregnant mice. The difference between humans and mice in the importance of Treg cells in late pregnancy phase may be explained by several reasons. One reason is the difference in placenta structure in humans and mice. Erlebacher et al. (2007) showed that maternal $CD8^+$ T cells recognize fetal/placental antigen leading to clonal deletion in the mid and late pregnancy period. They reported that antigen presentation commenced only at mid-gestation in association with endovascular invasion of placental trophoblasts and the hematogenous release of placental debris, and was associated with clonal deletion of $CD8^+$ T cells. Thus, while Treg cells appear to be necessary for implantation and the early phase of pregnancy, they may not have a critical role in the maintenance of pregnancy in the late gestation phase, because clonal deletion of $CD8^+$cells to paternal antigens might occur in the late pregnancy period.

Recent studies showed that abortion-prone CBA/J × DBA/2 mice present with a diminished number of $CD4^+CD25^+CTLA4^+IL\text{-}10^+$ Treg cells compared to normal pregnant mice (Zhu et al., 2005; Zenclussen et al., 2005). Zenclussen et al. (2005) reported that transfer of $CD4^+CD25^+$ Treg cells from normal pregnant, but not from non-pregnant, CBA/J mice completely prevented spontaneous abortion, suggesting that paternal antigen-specific $CD4^+CD25^+$ Treg cells induce paternal antigen-specific tolerance during pregnancy. By blocking ICAM-1/LFA-1 mediated intracellular adhesion events or the CD86/B7-ligand, the resorption rate in abortion-prone mice was decreased by expanding $CD4^+CD25^+$ Treg cells and by shifting cytokine profiles from Th1 predominance to a Th2 bias at the feto–maternal interface (Blois et al., 2005; Zhu et al., 2005). Robertson et al. (2009) reported that seminal fluid drives expansion of the Treg cell pool and induces tolerance to paternal alloantigens in mice. These observations are all consistent with paternal antigen specific Treg cells playing an important role for maintenance of allogeneic pregnancy during the implantation period and early pregnancy period.

These findings suggest that various mechanisms for immune tolerance interact with each other at the feto–maternal interface. In this study, we demonstrated that Treg cells play a central role in the maintenance of pregnancy at the implantation and early stage, but Treg cells may not have an essential role in the late stage of pregnancy in allogeneic pregnant mice.

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## References

Aluvihare, V.R., Kallikourdis, M., Betz, A.G., 2004. Regulatory T cells mediate maternal tolerance to the fetus. Nat. Immunol. 5, 266–271.

Arruvito, L., Sanz, M., Banham, A.H., Fainboim, L., 2007. Expansion of $CD4^+CD25^+$and $FOXP3^+$ regulatory T cells during the follicular phase of the menstrual cycle: implications for human reproduction. J. Immunol. 178, 2572–2578.

Baecher-Allan, C., Brown, J.A., Freeman, G.J., Hafler, D.A., 2001. $CD4^+CD25^{high}$ regulatory cells in human peripheral blood. J. Immunol. 167, 1245–1253.

Blois, S., Tometten, M., Kandil, J., Hagen, E., Klapp, B.F., Margni, R.A., Arck, P.C., 2005. Intercellular adhesion molecule-1/LFA-1 cross talk is a proximate mediator capable of disrupting immune integration and tolerance mechanism at the feto–maternal interface in murine pregnancies. J. Immunol. 174, 1820–1829.

Darmochwal-Kolarz, D., Saito, S., Rolinski, J., Tabarkiewicz, J., Kolarz, B., Leszczynska-Gorzelak, B., Oleszczuk, J., 2007. Activated T lymphocytes in pre-eclampsia. Am. J. Reprod. Immunol. 58, 39–45.

Darrasse-Jèze, G., Klatzmann, D., Charlotte, F., Salomon, B.L., Cohen, J.L., 2006. $CD4^+CD25^+$ regulatory/suppressor T cells prevent allogeneic fetus rejection in mice. Immunol. Lett. 102, 106–109.

Ebata, K., Shimizu, Y., Nakayama, Y., Minemura, M., Murakami, J., Kato, T., Yasumura, S., Takahara, T., Sugiyama, T., Saito, S., 2006. Immature NK cells suppress dendritic cell functions during the development of leukemia in a mouse model. J. Immunol. 176, 4113–4124.

Erlebacher, A., Vencato, D., Price, K.A., Zhang, D., Glimcher, L.H., 2007. Constraints in antigen presentation severely restrict T cell recognition of the allogeneic fetus. J. Clin. Invest. 117, 1399–1411.

Heikkinen, J., Möttönen, M., Alanen, A., Lassila, O., 2004. Phenotype characterization of regulatory T cells in the human decidua. Clin. Exp. Immunol. 136, 373–378.

Hoffmann, P., Ermann, J., Edinger, M., Fathman, C.G., Strober, S., 2002. Donor-type $CD4^+CD25^+$ regulatory T cells suppress lethal acute graft-versus-host disease after allogeneic bone marrow transplantation. J. Exp. Med. 196, 389–399.

Jasper, M.J., Tremellen, K.P., Robertson, S.A., 2006. Primary unexplained infertility is associated with reduced expression of the T-regulatory cell transcription factor Foxp3 in endometrial tissue. Mol. Hum. Reprod. 12, 301–308.

Jiang, S.P., Vacchio, M.S., 1998. Multiple mechanisms of peripheral T cell tolerance to the fetal "allograft". J. Immunol. 160, 3086–3090.

Jonuleit, H., Schmitt, E., Stassen, M., Tuettenberg, A., Knop, J., Enk, A.H., 2001. Identification and functional characterization of human

CD4+CD25+ T cells with regulatory properties isolated from peripheral blood. J. Exp. Med. 139, 1285–1294.
Karim, M., Bushell, A.R., Wood, K.J., 2002. Regulatory T cells in transplantation. Curr. Opin. Immunol. 14, 584–591.
Krey, G., Frank, P., Shaikly, V., Barrientos, G., Cordo-Russo, R., Ringel, F., Moschansky, P., Chernukhin, I.V., Metodiev, M., Fernández, N., Klapp, B.F., Arck, P.C., Blois, S.M., 2008. In vivo dendritic cell depletion reduces breeding efficiency, affecting implantation and early placental development in mice. J. Mol. Med. 86, 999–1011.
Lahl, K., Loddenkemper, C., Drouin, C., Freyer, J., Arnason, J., Eberl, G., Hamann, A., Wagner, H., Huehn, J., Sparwasser, T., 2007. Selective depletion of Foxp3+ regulatory T cells induces a scurfy-like disease. J. Exp. Med. 204, 57–63.
Levings, M.K., Sangregorio, R., Galbiati, F., Squadrone, S., de Waal Malefyt, R., Roncarolo, M.G., 2001. IFN-α and IL-10 induce the differentiation of human type 1 T regulatory cells. J. Immunol. 166, 5530–5539.
Lowenthal, J.W., Corthésy, P., Tougne, C., Lees, R., MacDonald, H.R., Nabholz, M., 1985. High and low affinity IL 2 receptors: analysis by IL 2 dissociation rate and reactivity with monoclonal anti-receptor antibody PC61. J. Immunol. 135, 3988–3994.
Lin, Y., Zhong, Y., Saito, S., Chen, Y., Shen, W., Di, J., Zeng, S., 2009. Characterization of natural killer cells in nonobese diabetic/severely compromised immunodeficient mice during pregnancy. Fertil. Steril. 91, 2676–2686.
Makrigiannakis, A., Zoumakis, E., Kalantaridou, S., Coutifaris, C., Margioris, A.N., Coukos, G., Rice, K.C., Gravanis, A., Chrousos, G.P., 2001. Corticotropin-releasing hormone promotes blastocyst implantation and early maternal tolerance. Nat. Immunol. 2, 1018–1024.
Ng, W.F., Duggan, P.J., Ponchel, F., Matarese, G., Lombardi, G., Edwards, A.D., Isaacs, J.D., Lechler, R.I., 2001. Human CD4+CD25+ cells: a naturally occurring population of regulatory T cells. Blood 98, 2736–2744.
Onizuka, S., Tawara, I., Shimizu, J., Sakaguchi, S., Fujita, T., Nakayama, E., 1999. Tumor rejection by *in vivo* administration of anti-CD25 (interleukin-2 receptor α) monoclonal antibody. Cancer Res. 59, 3128–3133.
Pasare, C., Medzhitov, R., 2003. Toll pathway-dependent blockade of CD4+CD25+ T cell-mediated suppression by dendritic cells. Science 299, 1033–1036.
Plaks, V., Birnberg, T., Berkutzki, T., Sela, S., BenYashar, A., Kalchenko, V., Mor, G., Keshet, E., Dekel, N., Neeman, M., Jung, S., 2008. Uterine DCs are crucial for decidua formation during embryo implantation in mice. J. Clin. Invest. 118, 3954–3965.
Robertson, S.A., Guerin, L.R., Bromfield, J.J., Branson, K.M., Ahlström, A.C., Care, A.S., 2009. Seminal fluid drives expansion of the CD4+CD25+ T regulatory cell pool and induces tolerance to paternal alloantigens in mice. Biol. Reprod. 80, 1036–1045.
Santner-Nanan, B., Peek, M.J., Khanam, R., Richarts, L., Zhu, E., Fazekas de St Groth, B., Nanan, R., 2009. Systemic increase in the ratio between Foxp3+ and IL-17-producing CD4+ T cells in healthy pregnancy but not in preeclampsia. J. Immunol. 183, 7023–7030.
Saito, S., Sasaki, Y., Sakai, M., 2005. CD4+CD25$^{high}$ regulatory T cells in human pregnancy. J. Reprod. Immunol. 65, 111–120.
Sakaguchi, S., 2004. Naturally arising CD4+ regulatory t cells for immunologic self-tolerance and negative control of immune responses. Annu. Rev. Immunol. 22, 531–562.
Sakaguchi, S., Sakaguchi, N., Asano, M., Itoh, M., Toda, M., 1995. Immunological self-tolerance maintained by activated T-cells expressing IL-2 receptor α-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various auto-immune diseases. J. Immunol. 155, 1151–1164.
Sasaki, Y., Sakai, M., Miyazaki, S., Higuma, S., Shiozaki, A., Saito, S., 2004. Decidual and peripheral blood CD4+CD25+ regulatory T cells in early pregnancy subjects and spontaneous abortion. Mol. Hum. Reprod. 10, 347–353.
Sasaki, Y., Darmochwal-Kolarz, D., Suzuki, D., Sakai, M., Ito, M., Shima, T., Shiozaki, A., Rolinski, J., Saito, S., 2007. Proportion of peripheral blood and decidual CD4+CD25$^{bright}$ regulatory T cells in pre-eclampsia. Clin. Exp. Immunol. 149, 139–145.
Somerset, D.A., Zheng, Y., Kilby, M.D., Sansom, D.M., Drayson, M.T., 2004. Normal human pregnancy is associated with an elevation in the immune suppressive CD4+CD25+ regulatory T-cell subset. Immunology 112, 38–43.
Stephens, L.A., Mottet, C., Mason, D., Powrie, F., 2001. Human CD4+CD25+ thymocytes and peripheral T cells have immune suppressive activity in vitro. Eur. J. Immunol. 31, 1247–1254.
Tafuri, A., Alferink, J., Möller, P., Hämmerling, G.J., Arnold, B., 1995. T cell awareness of paternal alloantigens during pregnancy. Science 270, 630–633.
Taylor, P.A., Noelle, R.J., Blazar, B.R., 2001. CD4+CD25+ immune regulatory cells are required for induction of tolerance to alloantigen via costimulatory blockade. J. Exp. Med. 193, 1311–1318.
Wood, K.J., Sakaguchi, S., 2003. Regulatory T cells in transplantation tolerance. Nat. Rev. Immunol. 3, 199–210.
Yang, H., Qiu, L., Chen, G., Ye, Z., Lü, C., Lin, Q., 2008. Proportional change of CD4+CD25+ regulatory T cells in decidua and peripheral blood in unexplained recurrent spontaneous abortion patients. Fertil. Steril. 89, 656–661.
Yang, Y., Huang, C.T., Huang, X., Pardoll, D.M., 2004. Persistent Toll-like receptor signals are required for reversal of regulatory T cell-mediated CD8 tolerance. Nat. Immunol. 5, 508–515.
Zenclussen, A.C., Gerlof, K., Zenclussen, M.L., Sollwedel, A., Bertoja, A., Ritter, T., Kostch, K., Leber, J., Volk, H.D., 2005. Abnormal T cell reactivity against paternal antigens in spontaneous abortion: adoptive transfer of pregnancy-induced CD4+CD25+ T regulatory cells prevents fetal rejection in a murine abortion model. Am. J. Pathol. 166, 811–822.
Zenclussen, A.C., 2005. CD4+CD25+ T regulatory cells in murine pregnancy. J. Reprod. Immunol. 65, 101–110.
Zhu, X.Y., Zhou, Y.H., Wang, M.Y., Jin, L.P., Yuan, M.M., Li, D.J., 2005. Blockade of CD86 signaling facilitates a Th2 bias at the maternal–fetal interface and expands peripheral CD4+CD25+ regulatory T cells to rescue abortion-prone fetuses. Biol. Reprod. 72, 338–345.

# 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)-$\gamma$, and tumor necrosis factor (TNF)-$\alpha$, 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)-$\beta$1 and IL-6, which activate signal transducer and activator of transcription 3 (Stat3) and induce the expression of the transcription factor retinoic acid-related orphan receptor gamma t (ROR$\gamma$t). On the other hand, the presence of TGF-$\beta$1 but not IL-6

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, Glostrup, Denmark) and diaminobenzidine (DAB; Sigma, Poole, 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, Burlingame, 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 (SSC) 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-$17^+$ cells were T cells (Fig. 1a). On the other hand, IL-$17^+$ 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-$17^+$ 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-$17^+$ 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-$17^+$ 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-$17^+$ cells in spontaneous abortion cases. IL-$17^+$ cells were distributed over the entire region of the decidua, the

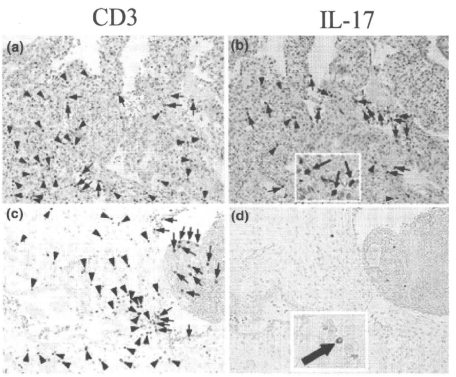


**Fig. 1** IL-17 expression in the decidua of spontaneous abortion cases: Serial paraffin sections of the decidua were stained with anti-CD3 (left panels) and anti-IL-17 (right panels) in spontaneous abortion (upper panels) or normal pregnancy (lower panels) cases of 7-week gestation. The expression 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).

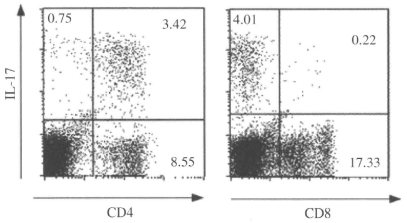


**Fig. 2** IL-17 expression in decidual lymphocytes: The intensity of IL-17 staining is shown on the *y*-axis, while the intensity of $CD4^+$ (left panel) or $CD8^+$ (middle panels) staining is plotted on the *x*-axis. The numbers represent the percentages of dots in each gated area.

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

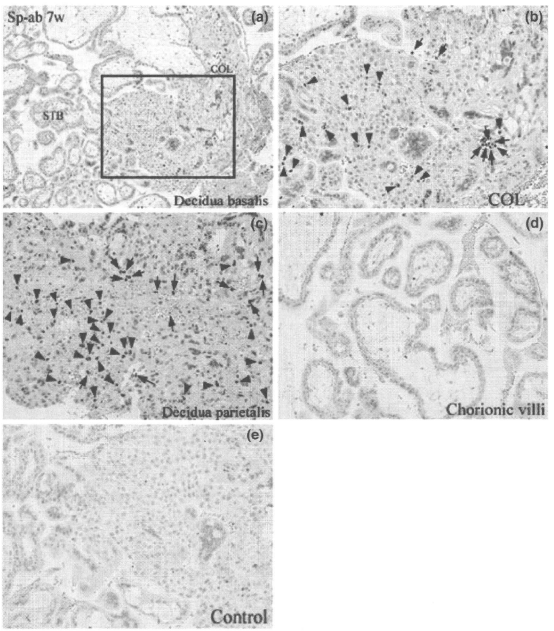


**Fig. 3** Distribution of IL-17$^+$ cells in inevitable abortion cases of 7-week gestation: Our immunohistochemical study showed the IL-17 expression in the decidua basalis (a), decidua parietalis (c), and villous (d) of inevitable abortion cases. Panel (b) shows the region outlined by a black line 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).

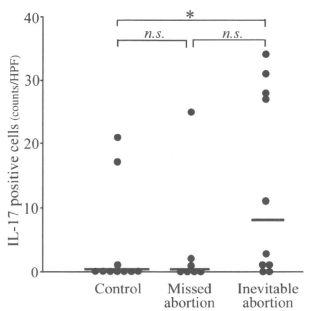


**Fig. 4** Comparison of the numbers of IL-17$^+$ cells in deciduas: The numbers of IL-17$^+$ cells in deciduas from normal pregnancy, missed abortion, and inevitable abortion cases. The bars indicate the median values. *$P < 0.05$.

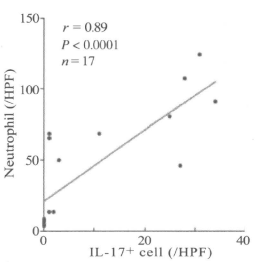


**Fig. 5** Correlation between the numbers of IL-17$^+$ cells and neutrophils: A scatter graph was constructed between the numbers of IL-17$^+$ cells (*X*-axis) and neutrophils (*Y*-axis) in spontaneous abortion cases. The coefficient of correlation (*r*) is shown on the upper side of the graph. The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

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## References

1 Mosmann TR, Coffman RL: TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu Rev Immunol* 1989; 7:145–173.
2 Hill JA, Polgar K, Anderson DJ: T-helper 1-type immunity to trophoblast in women with recurrent spontaneous abortion. *JAMA* 1995; 273:1933–1936.
3 Piccinni MP, Beloni L, Livi C, Maggi E, Scarselli G, Romagnani S: Defective production of both leukemia inhibitory factor and type 2 T-helper cytokines by decidual T cells in unexplained recurrent abortions. *Nat Med* 1998; 4:1020–1024.
4 Bates MD, Quenby S, Takakuwa K, Johnson PM, Vince GS: Aberrant cytokine production by peripheral blood mononuclear cells in recurrent pregnancy loss? *Hum Reprod* 2002; 17:2439–2444.
5 Chaouat G: Innately moving away from the Th1/Th2 paradigm in pregnancy. *Clin Exp Immunol* 2003; 131:393–395.
6 Park H, Li Z, Yang XO, Chang SH, Nurieva R, Wang YH, Wang Y, Hood L, Zhu Z, Tian Q, Dong C: A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nat Immunol* 2005; 6:1133–1141.
7 Harrington LE, Hatton RD, Mangan PR, Turner H, Murphy TL, Murphy KM, Weaver CT: Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nat Immunol* 2005; 6:1123–1132.
8 Komiyama Y, Nakae S, Matsuki T, Nambu A, Ishigame H, Kakuta S, Sudo K, Iwakura Y: IL-17 plays an important role in the development of experimental autoimmune encephalomyelitis. *J Immunol* 2006; 177:566–573.
9 Weaver CT, Hatton RD, Mangan PR, Harrington LE: IL-17 family cytokines and the expanding diversity of effector T cell lineages. *Annu Rev Immunol* 2007; 25:821–852.
10 Aluvihare VR, Kallikourdis M, Betz AG: Regulatory T cells mediate maternal tolerance to the fetus. *Nat Immunol* 2004; 5:266–271.
11 Sasaki Y, Sakai M, Miyazaki S, Higuma S, Shiozaki A, Saito S: Decidual and peripheral blood CD4+ CD25+ regulatory T cells in early pregnancy subjects and spontaneous abortion cases. *Mol Hum Reprod* 2004; 10:347–353.
12 Jasper MJ, Tremellen KP, Robertson SA: Primary unexplained infertility is associated with reduced expression of the T-regulatory cell transcription factor Foxp3 in endometrial tissue. *Mol Hum Reprod* 2006; 12:301–308.
13 Loong CC, Hsieh HG, Lui WY, Chen A, Lin CY: Evidence for the early involvement of interleukin 17 in human and experimental renal allograft rejection. *J Pathol* 2002; 197:322–332.
14 Lindén A, Laan M, Anderson GP: Neutrophils, interleukin-17A and lung disease. *Eur Respir J* 2005; 25:159–172.
15 Kumada T, Tsuneyama K, Hatta H, Ishizawa S, Takano Y: Improved 1-h rapid immunostaining method using intermittent microwave irradiation: practicability based on 5 years application in Toyama Medical and Pharmaceutical University Hospital. *Mod Pathol* 2004; 17:1141–1149.
16 Ito M, Nakashima A, Ina S, Okabe M, Bac ND, Yoneda S, Shiozaki A, Tsuneyama K, Nikaido T, Hidaka T, Saito S: Interleukin-17 (IL-17) and tumor necrosis factor α synergistically increase IL-8 production by amniotoic methenchymal cells in preterm delivery cases with chorioamnionitis. *J Reprod Immunol* 2010; 84:75–85.
17 Stray-Pedersen B, Stray-Pedersen S: Etiologic factors and subsequent reproductive performance in 195 couples with a prior history of habitual abortion. *Am J Obstet Gynecol* 1984; 148:140–146.
18 Matsuzaki G, Umemura M: Interleukin-17 as an effector molecule of innate and acquired immunity against infections. *Microbiol Immunol* 2007; 51:1139–1147.
19 Korn T, Oukka M, Kuchroo V, Bettelli E: Th17 cells: effector T cells with inflammatory properties. *Semin Immunol* 2007; 19:362–371.
20 Hirata T, Osuga Y, Hamasaki K, Yoshino O, Ito M, Hasegawa A, Takemura Y, Hirota Y, Nose E, Morimoto C, Harada M, Koga K, Tajima T, Saito S, Yano T, Taketani Y: Interleukin (IL)-17A stimulates IL-8 secretion, cyclooxygensase-2 expression, and cell proliferation of endometriotic stromal cells. *Endocrinology* 2008; 149:1260–1267.
21 Nakashima A, Ito M, Yoneda S, Shiozaki A, Hidaka T, Saito S: Circulating and decidual Th17 cell levels in

healthy pregnancy. *Am J Reprod Immunol* 2010; 63:104–109.

22 Pongcharoen S, Somran J, Sritippayawan S, Niumsup P, Chanchan P, Butkhamchot P, Tatiwat P, Kunngurn S, Searle RF: Interleukin-17 expression in the human placenta. *Placenta* 2007; 28:59–63.

23 Pongcharoen S, Niumsup P, Sanguansermsri D, Supalap K, Butkhamchot P: The effect of interleukin-17 on the proliferation and invasion of JEG-3 human choriocarcinoma cells. *Am J Reprod Immunol* 2006; 55:291–300.

24 Nakashima A, Shiozaki A, Myojo S, Ito M, Tatematsu M, Sakai M, Takamori Y, Ogawa K, Nagata K, Saito S: Granulysin produced by uterine natural killer cells induces apoptosis of extravillous trophoblasts in spontaneous abortion. *Am J Pathol* 2008; 173:653–664.

25 Starnes T, Robertson MJ, Sledge G, Kelich S, Nakshatri H, Broxmeyer HE, Hromas R: IL-17F, a novel cytokine selectively expressed in activated T cells and monocytes, regulates angiogenesis and endothelial cell cytokine production. *J Immunol* 2001; 167:4137–4140.

26 Bulmer JN, Williams PJ, Lash GE: Immune cells in the placental bed. *Int J Dev Biol* 2010; 54:281–294.

# Midline uterine defect size is correlated with miscarriage of euploid embryos in recurrent cases

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**Objective:** To compare subsequent pregnancy outcomes after two or more miscarriages in patients with and without congenital uterine anomalies.
**Design:** Case-control study.
**Setting:** Nagoya City University Hospital.
**Patient(s):** A total of 42 patients with a bicornuate or septate uterus and 1528 with normal uteri.
**Intervention(s):** No surgery.
**Main Outcome Measure(s):** The cumulative success rate for birth, abnormal chromosome karyotype rate in aborted concepti, and the predictive values of the height of the defect/length of the remaining uterine cavity ratio (D/C ratio).
**Result(s):** Of the total of 1676 patients, 54 (3.2%) had congenital uterine anomalies; 25 (59.5%) of the 42 patients with a bicornuate or septate uterus had a successful first pregnancy after examination, while this was the case for 1096 (71.7%) of the 1528 with normal uteri. There was no difference in the cumulative live-birth rate (78.0% and 85.5%) within the follow-up period. However, the rates for an abnormal chromosome karyotype in aborted concepti in cases with and without uterine anomalies were 15.4% (two of 13) and 57.5% (134 of 233), respectively, with the latter being significantly higher. The D/C ratio in the miscarriage group was also significantly greater than that for the live-birth group.
**Conclusion(s):** Congenital uterine anomalies have a negative impact on reproductive outcome in couples with recurrent miscarriage and are associated with further miscarriage with a normal embryonic karyotype. The D/C ratio was found to have a predictive value for further miscarriages in recurrent cases. (Fertil Steril® 2010;93:1983–8. ©2010 by American Society for Reproductive Medicine.)

**Key Words:** Bicornuate uterus, congenital uterine anomaly, recurrent miscarriage, septate uterus

Established causes of recurrent miscarriages are antiphospholipid antibodies (aPL), uterine anomalies, and chromosomal abnormalities in the embryo (1–3). Abnormal chromosomes in either partner, particularly translocations, are also risk factors (4). Regarding uterine anomalies, Raga et al. reported that patients (6.3%, 54 of 868; $P<.05$) with a history of two or more miscarriages had a significantly elevated incidence of Mullerian anomalies compared with fertile (3.8%, 49 of 1289) and sterile (2.4%, 25 of 1024) cases (2). The frequency of congenital uterine anomalies has been reported to be between 1.8% and 37.6% in women with a history of recurrent miscarriage, the variation largely depending on the methods of selection and criteria for diagnosis (5–7).

Thus, affected patients are offered surgery in an attempt to restore the uterine anatomy (8–16). The conclusion is that operations can increase successful pregnancies, but to our knowledge there have been no prospective studies comparing pregnancy outcomes between cases with and without surgery in patients with a history of recurrent miscarriage. Lee et al. reported a preoperative pregnancy loss rate of 77.4%, a 18.2% miscarriage rate, and a 77.3% uncomplicated delivery rate after hysteroscopic septum resection (14). However, it is inappropriate to simply make comparisons before and after surgery because the miscarriage rate before examination might be 100% but the subsequent success rate is never 0. The subsequent live-birth rate is expected to be 72% in recurrent miscarriage patients without abnormal chromosomes in either partner (17) and decreases with the number of previous miscarriages (3).

Information concerning the prognosis in women with congenital uterine anomalies with a history of recurrent miscarriage is limited. The present study was therefore conducted to assess the subsequent live-birth rate, comparing pregnancy outcome between cases with and without bicornis or septum in individuals with a history of recurrent miscarriage.

## PATIENTS AND METHODS

We conducted a case-control study. We studied 1676 patients with a history of two or more (2–12) consecutive miscarriages whose subsequent pregnancies were ascertained at least once in our medical records. Hysterosalpingography (HSG), chromosome analysis for both partners, determination of aPL,

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including lupus anticoagulant and β2-glycoprotein I dependent anticardiolipin antibodies (18), and blood tests for hyperthyroidism, diabetes mellitus, and hyperprolactinemia were performed for all patients before subsequent pregnancy. All patients were examined between 1986 and 2007 at Nagoya City University Hospital.

Laparoscopy/laparotomy and/or magnetic resonance imaging (MRI) were performed to ascertain the type of anomaly (investigating both the uterine cavity and the external uterine contours) in accordance with the American Fertility Society classification of Mullerian anomalies (19–21). Tompkin's index was used to distinguish between arcuate uterus and mild septate or bicornuate uterus (22). A Tompkin's index >25% was the criterion for septate or bicornuate uterus. Patients desiring surgical treatment before subsequent pregnancies underwent a Jones metroplasty, Strassman metroplasty, or hysteroscopic transcervical resection (TCR; 8–10).

All pregnancy outcomes of 1676 patients were examined. Patients with at least one kind of aPL were treated with low-dose aspirin and heparin combined therapy. Gestational age was calculated from basal body temperature charts. Ultrasound was performed once or twice a week from 4 to 8 weeks' gestation. Dilation and curettage was performed on all patients diagnosed with miscarriage, and the karyotypes of aborted conceptuses were determined with the use of a standard G-banding technique. The study was approved by the Research Ethics Committee at Nagoya City University Medical School.

In the present study, [1] the prevalence of clear congenital uterine malformations such as septate uterus, bicornuate uterus, unicornuate uterus, and didelphys was examined; [2] the first pregnancy outcome after systematic examination for recurrent miscarriage was determined for both septate and bicornuate uterus cases, comparing patients with or without anomalies; [3] all pregnancy outcomes after systematic examination were also assessed, and the final live-birth rate/patient was calculated; [4] abnormal karyotype rates for aborted concepti at the first miscarriage after the ascertainment of uterine abnormalities were also compared between patients with and without congenital uterine anomalies; [5] the height of the defect/length of the remaining uterine cavity (D/C) ratios were calculated in cases with bicornuate and septate uterine and compared between miscarriages and live birth at the subsequent first pregnancy. We also ascertained whether the D/C ratio has predictive value for further miscarriage in recurrent miscarriage cases.

The analysis was carried out using the SAS system (SAS Institute Inc., Cary, NC) with receiver operating curve (ROC) analysis and logistic regression. $P<.05$ was considered statistically significant.

## RESULTS

### Baseline Characteristics

One thousand six hundred seventy-six patients became pregnant after systematic examination for recurrent miscarriages. Of this total, 54 (3.2%) had congenital uterine anomalies, 38 with partial bicornis unicolli, 10 with a septum, five with a unicornis, and one with a didelphys. None of them had hypoplasis/agenesis or diethylstilboestrol (DES) drug-related anomalies. Two patients with a septate uterus and a bicornuate uterus also had translocations in either partner. The 94 patients who had structural chromosome abnormalities, including 73 translocations, in either partner, were excluded from the analysis.

One thousand five hundred twenty-eight patients had neither congenital uterine anomalies nor an abnormal chromosome karyotype in either partner; 75 patients exhibited persistent aPL and were treated with low-dose aspirin and heparin combined therapy.

One of the two patients with bicornuate uteri underwent a Jones metroplasty, and the other underwent a Strassman metroplasty (8, 9). One patient with a septum also received a Jones metroplasty, and hysteroscopic TCR was performed for the other four patients with septate uteri.

We compared pregnancy outcomes between 42 patients with septate or bicornuate uteri not undergoing surgery and 1528 patients without uterine anomaly. We found no differences in baseline characteristics between the two groups (Table 1).

### Pregnancy Outcome

Subsequent pregnancy outcomes are summarized in Table 2. Twenty-five of the 42 patients with a septate or bicornuate uterus (59.5%) treated without any kind of surgery had a successful outcome, while this was the case for 1096 (71.7%) of the 1528 without congenital uterine anomalies at the subsequent first pregnancy ($P=.084$). Four of five patients with a septate uterus and 21 of 37 patients with a bicornuate uterus gave birth to live babies. There was one case with a bicornuate uterus who suffered from uterine rupture in the first trimester because of the limited capacity.

One patient received surgery after further miscarriage. Thus, 32 (78.0%) of 41 patients and 1307 (85.5%) of 1528 patients with and without uterine anomalies could cumulatively have a live baby within the follow-up period ($P$ = not significant). Live-birth rates of patients with congenital uterine anomalies tended to be lower both at the first pregnancy after ascertainment and cumulatively. Final live-birth rates/person are also shown in Table 2.

Furthermore, rates for an abnormal chromosome karyotype in aborted concepti in cases with and without uterine anomalies were 15.4% (two of 13) and 57.5% (134 of 233), respectively, at the first pregnancy after ascertainment of uterine anomalies, the difference being highly significant (Fisher's exact probability test, $P=.006$).

One of five patients with a unicornuate uterus succeeded in having a baby at the first pregnancy after examination, and four of five could have a baby, cumulatively. The patient with didelphys also succeeded at the first pregnancy after examination.

**TABLE 1**

**Baseline characteristics of patients with and without congenital uterine anomalies.**

| | Patients with anomalies (n = 42) | Patients without anomalies (n = 1528) | *P* value |
|---|---|---|---|
| Maternal age, y | | | |
| Mean (SD) | 31.5 (3.5) | 31.1 (4.3) | NS |
| Median (interquartile range) | 31 (29) | 31 (28) | NS |
| Number of previous miscarriages | | | |
| 2 | 17 (40.5) | 765 (50.1) | |
| 3 | 18 (42.9) | 537 (35.1) | |
| 4 | 7 (16.7) | 136 (8.9) | |
| 5 or more | 0 | 90 (5.9) | .085 |
| Mean (SD) | 2.74 (0.77) | 2.77 (1.12) | NS |
| Median (interquartile range) | 3 (2) | 2 (2) | NS |
| No. of previous live births | | | |
| 0 | 37 (88.1) | 1328 (86.9) | |
| 1 | 4 (9.5) | 186 (12.2) | |
| 2 or more | 1 (2.4) | 14 (0.9) | NS |
| Mean (SD) | 0.1 | 0.14 (0.37) | NS |
| No. of previous stillbirths | | | |
| 0 | 40 (95.2) | 1491 (97.6) | |
| One or more | 2 (4.8) | 37 (2.4) | NS |

*Note:* Values are numbers (percentages) of patients unless otherwise specified.

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### Predictive Value for the D/C Ratio

Mean values (SD) for the D/C ratio in the miscarriage and live-birth groups were 0.8332 (0.3974) and 0.4776 (0.2745), respectively ($P$=.0057, 95% confidence interval [CI]; 0.1115–0.5998). When two miscarriage cases caused by an abnormal embryonic karyotype were excluded, the value for the D/C ratio in the miscarriage group was also significantly higher than in the live-birth group ($P$=.0051). Mean (SD) age and number of previous miscarriages for the 15 patients whose subsequent pregnancy ended in miscarriage and the 17 patients who experienced live births were 31.5 (3.0) versus 31.5 (3.8) and 2.76 (0.75) versus 2.72 (0.79), respectively ($P$ = not significant). Ten patients were excluded because HSG films were not available.

The ROC curve is shown in Figure 1. From the figure, the cutoff value would be appropriate somewhere between 0.59 and 0.64, giving the sensitivity and specificity around 0.75–0.80. The area under the ROC curve, meaning the total diagnostic accuracy of the D/C ratio on live birth, was 0.808. From the logistic regression, the D/C ratio was found to be an independent risk factor on the failure of live birth after adjusting for age and previous number of miscarriages. The odds ratio for the 0.1 increment of D/C ratio was 1.42 (95% CI, 1.06–1.91).

## DISCUSSION

In the present study, the live-birth rate of patients with congenital uterine anomalies tended to be lower, both at the first pregnancy after ascertainment and cumulatively, than that of patients with a normal uterus, although the differences were not significant. Congenital uterine anomalies were associated with miscarriages with a normal embryonic karyotype. Thus, congenital uterine anomalies impacted the progression of normal pregnancies.

Salim et al. earlier found no significant difference in the relative frequency of various anomalies or depth of fundal distortion as determined by three-dimensional (3D) ultrasound between women with and without a history of recurrent miscarriage, although abnormalities in uterine anatomy were more severe in women with a history of recurrent miscarriages (23). In this context, the finding in the present study that the D/C ratio is a predictor of further miscarriage in recurrent cases is clearly of interest.

However, 59.5% and 78.0% of our patients with a septate or bicornuate uterus without any kind of surgery could have a baby at the first pregnancy or cumulatively. Several studies concerning obstetric outcome after removal of a uterine septum have been reported (10–16). Lee et al. described a 77.3% uncomplicated delivery rate after hysteroscopic septum resection (14). Kormayos et al. compared pregnancy outcome after removal of septum between cases with and without a residual septum in patients with a history of two or three miscarriages and concluded that the live-birth rate in cases with no remnant was significantly higher than that in cases with a remnant (15). However, the live-birth rate for patients undergoing first hysteroscopy was 35.1% (33 of 94), and the

**TABLE 2**

**Successful reproductive outcome after examination of uterine anomalies in patients with recurrent miscarriage.**

| | Success rate per pregnancy | | | | | | Cumulative success rate | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | With anomalies (n = 42) | Bicornuate | Septum | Without anomalies (n = 1528) | Difference in % | *P* value | With anomalies (n = 41)[a] | Without anomalies (n = 1528) | Difference in % | *P* value |
| Pregnancy after the ascertainment of uterine anomaly | | | | | | | | | | |
| First | 25/42 (59.5)[b] | 21/37 (56.8) | 4/5 (80.0) | 1096/1528 (71.7) | −12.2 | .084 | 25 (61.0) | 1096 (71.7) | −10.7 | .133 |
| Second | 5/9 (55.6) | 4/8 (50.0) | 2/2 (100) | 166/275 (60.4)[c] | −4.8 | .772 | 30 (73.2) | 1262 (82.6) | −9.4 | .119 |
| Third | 2/2 (100) | 2/2 (100) | | 38/69 (55.0) | +45.0 | .207 | 32 (78.0) | 1300 (85.1) | −7.1 | .215 |
| Fourth | | | | 4/18 (22.2) | | | | 1304 (85.3) | | |
| Fifth | | | | 3/9 (33.3) | | | | 1307 (85.5) | | |
| Sixth | | | | 0/6 (0) | | | | 1307 (85.5) | | |
| Final follow up | | | | | | | 32 (78.0) | 1307 (85.5) | −7.5 | |

*Note:* Values are numbers (percentages) of couples. Success rate is defined as the live birth.

[a] One case underwent surgery between the first and second pregnancy after the ascertainment of an anomaly, thus this case was excluded from the cumulative analysis.

[b] Comparison was performed between patients both with anomalies and with normal uterus.

[c] Cases who could succeed in the first pregnancy were excluded from the analysis of the second and subsequent pregnancies.

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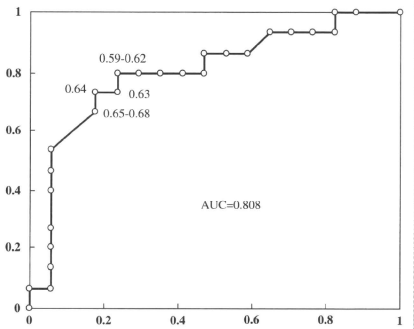
**FIGURE 1**

ROC analysis of D/C ratio.

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cumulative live-birth rate after one or two metroplasties was 54.3% (51 of 94). Both live-birth rates were lower than that without surgery in the present study. The benefits of surgical correction (open and hysteroscopic) on pregnancy outcome have yet to be assessed in a randomized trial, but the D/C ratio might be useful in deciding who should be selected.

### Limitations

In the present study, clear uterine malformations such as septate, bicornuate, or unicornuate uterus and didelphys were found in 3.2% of patients. The prevalence of clear congenital uterine anomalies in patients with a history of recurrent miscarriages has been reported to be 1.8%–20.1% with the arcuate uterus excluded (5–7) and thus higher than the 2.2% documented for fertile women (28 of 1289) (2). Minor malformations like arcuate uterus do not appear to have any impact on reproduction (2), and therefore we here excluded cases with this anomaly.

HSG is the diagnostic modality that has most often led to a tentative diagnosis of congenital anomalies (19), but when used alone it cannot distinguish between a septate and a bicornuate uterus. Thus laparoscopy has hitherto been needed for a final diagnosis. The advent of sonohysterography, MRI (20), and 3D ultrasound now allows for accurate differential diagnosis (21), although distinguishing an arcuate from a mildly subseptate or bicornuate uterus still remains difficult.

It is important to distinguish between the bicornuate uterus and the septate uterus, especially regarding the selection of surgical methods because TCR should not be performed for the former. We here ascertained the type of anomaly to study the prevalence in accordance with the American Fertility Society classification of Mullerian anomalies. Woelfer et al. proved new 3D criteria by which a bicornuate uerus can be distinguished from a septate uterus when a fundal indentation >10 mm dividing the two cornua is detectable (21). Using 3D ultrasound, it has been found that the septate uterus has the higher incidence. The criteria are useful before deciding on using TCR for the septum. It is difficult to examine the significance of the distinction between bicornuate and septate uteri because of the absence of internationally established criteria, although we have given the live-birth rate for each anomaly in Table 2. Thus we focused not on type of anomalies but rather on the D/C ratio. In addition, the sample size in the anomaly group was too small to allow any conclusion when we distinguished between the two groups.

While we examined 1676 patients who became pregnant at least one time in the present study, we failed to follow up all those who received systemic examination for causes of recurrent miscarriage at our hospital because some lived at a long distance. Some patients might become infertile after miscarriage. A prospective case-control study should therefore be conducted to compare live-birth rates between patients with and without surgery, including consideration of the infertile rate.

### Conclusion

Congenital uterine anomalies have a negative impact on reproductive outcome in couples with recurrent miscarriage, being particularly associated with normal embryonic karyotype miscarriages. The height of the defect/length of the remaining uterine cavity ratio, the D/C ratio, has independent predictive value for further miscarriage in recurrent cases. Comparison of cases of anomalies with and without surgery is needed in future recurrent miscarriage studies.

## REFERENCES


1. Farquharson RG, Pearson JF, John L. Lupus anticoagulant and pregnancy management. Lancet 1984;28:228–9.
2. Raga F, Bauset C, Remohi J, Bonilla-Musoles F, Simon C, Pellicer A. Reproductive impact of congenital Mullerian anomalies. Hum Reprod 1997;12:2277–81.
3. Ogasawara M, Aoki K, Okada S, Suzumori K. Embryonic karyotype of abortuses in relation to the number of previous miscarriages. Fertil Steril 2000;73:300–4.
4. Sugiura-Ogasawara M, Ozaki Y, Sato T, Suzumori N, Suzumori K. Poor prognosis of recurrent aborters with either maternal or paternal reciprocal translocation. Fertil Steril 2004;81:367–3.
5. Makino T, Umeuchi M, Nakada K, Nozawa S, Iizuka R. Incidence of congenital uterine anomalies in repeated reproductive wastage and prognosis for pregnancy after metroplasty. Int J Fertil 1992;37:167–70.
6. Cliford K, Rai R, Watson H, Regan L. An informative protocol for the investigation of recurrent miscarriage: preliminary experience of 500 consecutive cases. Hum Reprod 1994;9:1328–32.
7. Acien P. Incidence of Mullerian defects infertile and infertile women. Hum Reprod 1997;12:1372–6.
8. Jones HW, Jones GE. Double uterus as an etiological factor in repeated abortion: indications for surgical repair. Am J Obstet Gynecol 1953;65: 325–39.
9. Strassmann EO. Operations for double uterus and endometrial atresia. Clin Obstet Gynecol 1961;4:240.