Table 3 Distribution of variables according to age

Variables	<60 years	($n = 67$)	≥60 years	($n = 43$)	*P*-value (×2)
PLN					
(+)	21	(41%)	15	(27%)	0.96
(−)	30	(59%)	41	(73%)	
PALN					
(+)	15	(23%)	9	(20%)	0.67
(−)	50	(77%)	36	(80%)	
FIGO stage					
I	20	(33%)	16	(36%)	0.65
II–III	41	(67%)	28	(64%)	
Histological type					
EA	43	(69%)	41	(89%)	0.019
not EA	19	(31%)	5	(11%)	
FIGO grade					
1	25	(50%)	16	(47%)	0.81
2	14	(28%)	11	(32%)	
3	11	(22%)	7	(21%)	
Risk group					
High	51	(80%)	25	(57%)	0.017
Intermediate–high	12	(19%)	18	(41%)	
Intermediate–low	1	(1%)	1	(2%)	
Chemotherapy					
With	30	(49%)	16	(37%)	0.21
Without	31	(51%)	27	(63%)	
Depth					
a–b†	10	(19%)	8	(18%)	0.27
c–d‡	44	(81%)	36	(82%)	

†<50% myometrial invasion. ‡>50% myometrial invasion. DFS, disease-free survival; EA, endometrioid adenocarcinoma; FIGO, International Federation of Gynecology and Obstetrics; OS, overall survival; PALN, para-aortic lymph node; PLN, pelvic lymph node.

were only 15 examples for stage II, and the number of cases might not be sufficient to analyze treatment results. On the other hand, there were 55 examples of stage III, which constitutes an excellent OS rate. A phase III randomized trial showed improved survival with the use of chemotherapy for stage III and IV endometrial cancer.[20,21] However, pelvic and abdominal failure rates were alarmingly high, which appears to be persuasive for the integration of radiation and chemotherapy as performed in our institution.

According to our multivariate analysis of DFS, being a senior citizen is in itself an independent risk factor. More intensive treatment may be necessary for senior citizens than for young people. A total dose of approximately 50 Gy in PORT has already been prescribed, and because any further dose increase is difficult, the inclusion of postoperative chemotherapy can be expected. According to a recent Japanese Gynecologic Oncology Group study,[22] adjuvant CAP chemotherapy may be a useful alternative to PORT for intermediate-risk endometrial cancer. Moreover, adjuvant vaginal high-dose-rate brachytherapy alone may be a safe and effective alternative to pelvic external beam PORT for surgical early stage endometrial cancer.[23,24]

References

1. Kosary CL. FIGO stage, histology, histologic grade, age and race as prognostic factors in determining survival for cancers of the female gynecological system: An analysis of 1973–1987 SEER cases of cancers of the endometrium, cervix, ovary, vulva, and vagina. *Semin Surg Oncol* 1994; 10: 31–46.
2. Zaino RJ, Kurman RJ, Diana KL, Morrow CP. Pathologic models to predict outcome for women with endometrioid adenocarcinoma: The importance of the distinction between surgical stage and clinical stage – a Gynecologic Oncology Group study. *Cancer* 1996; 77: 1115–1121.
3. Abeler VM, Kjørstad KE. Endometrial adenocarcinoma in Norway. A study of a total population. *Cancer* 1991; 67: 3093–3103.
4. Irwin C, Levin W, Fyles A, Pintilie M, Manchul L, Kirkbride P. The role of adjuvant radiotherapy in carcinoma of the endometrium: results in 550 patients with pathologic stage I disease. *Gynecol Oncol* 1998; 70: 247–254.

© 2010 The Authors
Journal compilation © 2010 Japan Society of Obstetrics and Gynecology

5. Poulsen MG, Roberts SJ. Prognostic variables in endometrial carcinoma. *Int J Radiat Oncol Biol Phys* 1987; 13: 1043–1052.
6. Mundt AJ, Waggoner S, Yamada D, Rotmensch J, Connell PP. Age as a prognostic factor for recurrence in patients with endometrial carcinoma. *Gynecol Oncol* 2000; 79: 79–85.
7. Grigsby PW, Perez CA, Kuten A *et al.* Clinical stage I endometrial cancer: Prognostic factors for local control and distant metastasis and implications of the new FIGO surgical staging system. *Int J Radiat Oncol Biol Phys* 1992; 22: 905–911.
8. Lindahl B, Ranstam J, Willén R. Five year survival rate in endometrial carcinoma stages I-II: Influence of degree of tumour differentiation, age, myometrial invasion and DNA content. *Br J Obstet Gynaecol* 1994; 101: 621–625.
9. Alektiar KM, McKee A, Venkatraman E *et al.* Intravaginal high-dose-rate brachytherapy for Stage IB (FIGO Grade 1, 2) endometrial cancer. *Int J Radiat Oncol Biol Phys* 2002; 53: 707–713.
10. Samet J, Hunt WC, Key C, Humble CG, Goodwin JS. Choice of cancer therapy varies with age of patient. *JAMA* 1986; 255: 3385–3390.
11. Schrag D, Cramer LD, Bach PB, Begg CB. Age and adjuvant chemotherapy use after surgery for stage III colon cancer. *J Natl Cancer Inst* 2001; 93: 850–857.
12. Alektiar KM, Venkatraman E, Abu-Rustum N, Barakat RR. Is endometrial carcinoma intrinsically more aggressive in elderly patients? *Cancer* 2003; 98: 2368–2377.
13. Creutzberg CL, Van Putten WL, Koper PC *et al.* Surgery and postoperative radiotherapy versus surgery alone for patients with stage-1 endometrial carcinoma: Multicentre randomized trial. PORTEC Study Group. Post Operative Radiation Therapy in Endometrial Carcinoma. *Lancet* 2000; 355: 1404–1411.
14. Jolly S, Vargas CE, Kumar T *et al.* The impact of age on long-term outcome in patients with endometrial cancer treated with postoperative radiation. *Gynecol Oncol* 2006; 103: 87–93.
15. Lee NK, Cheung MK, Shin JY *et al.* Prognostic factors for uterine cancer in reproductive-aged women. *Obstet Gynecol* 2007; 109: 655–662.
16. Farley JH, Nycum LR, Birrer MJ, Park RC, Taylor RR. Age-specific survival of women with endometrioid adenocarcinoma of the uterus. *Gynecol Oncol* 2000; 79: 86–89.
17. Manton KG, Vaupel JW. Survival after the age of 80 in the United States, Sweden, France, England, and Japan. *N Engl J Med* 1995; 333: 1232–1235.
18. Taeuber CM. Sixty-five plus in America. Current Population Reports. Special Studies. Washington, DC: US Department of Commerce, Economics and Statistics Administration, Bureau of the Census, 1996; publication no. P23–173RV.
19. The Cancer Committee of the Japan Society of Gynecology. [The 41st treatment annual report. 5-year result of treatment started in 1993 for cervical and endometrial carcinoma.] *J Jpn Soc Obstet Gynecol* 2008; 60: 1876–1901. (In Japanese.)
20. Randall ME, Brunetto VL, Muss H. Whole abdominal radiotherapy versus combination doxorubicin-cisplatin chemotherapy in advanced endometrial carcinoma: A randomized phase III trial of the Gynecologic Oncology Group. *Proc Am Soc Clin Oncol* 2003; 22: 2.
21. Randall ME, Spirtos NM, Dvoretsky P. Whole abdominal radiotherapy versus combination chemotherapy with doxorubicin and cisplatin in advanced endometrial carcinoma (phase III): Gynecologic Oncology Group Study no. 122. *J Natl Cancer Inst Monogr* 1995; 19: 13–15.
22. Susumu N, Sagae S, Udagawa Y *et al.* Japanese Gynecologic Oncology Group.Randomized phase III trial of pelvic radiotherapy versus cisplatin-based combined chemotherapy in patients with intermediate- and high-risk endometrial cancer: A Japanese Gynecologic Oncology Group study. *Gynecol Oncol* 2008; 108: 226–233.
23. Jolly S, Vargas C, Kumar T *et al.* Vaginal brachytherapy alone: An alternative to adjuvant whole pelvis radiation for early stage endometrial cancer. *Gynecol Oncol* 2005; 97: 887–892.
24. Solhjem MC, Petersen IA, Haddock MG. Vaginal brachytherapy alone is sufficient adjuvant treatment of surgical stage I endometrial cancer. *Int J Radiat Oncol Biol Phys* 2005; 62: 1379–1384.

© 2010 The Authors
Journal compilation © 2010 Japan Society of Obstetrics and Gynecology

Med Oncol
DOI 10.1007/s12032-010-9511-6

ORIGINAL PAPER

Aromatase inhibitor anastrozole as a second-line hormonal treatment to a recurrent low-grade endometrial stromal sarcoma: a case report

Keiko Shoji · Katsutoshi Oda · Shunsuke Nakagawa · Kei Kawana · Toshiharu Yasugi · Yuji Ikeda · Yutaka Takazawa · Shiro Kozuma · Yuji Taketani

Received: 18 January 2010 / Accepted: 17 March 2010
© Springer Science+Business Media, LLC 2010

Abstract Low-grade endometrial stromal sarcoma (ESS) is a rare neoplasm and is generally an indolent tumor with estrogen and progesterone receptors. Objective responses by hormonal treatment with progestin or aromatase inhibitor have been reported, however, long-term management of this disease could be difficult if it becomes refractory to one of these hormonal therapies. A 34-year-old woman was diagnosed with stage I low-grade ESS at the time of hysterectomy for presumed uterine fibroma. Five years later, she recurred with multiple tumors in the lower abdomen. After an optimal surgery, she was free from progression for 6 years with progestin treatment (medroxyprogesterone acetate: MPA, 200–600 mg daily). Thereafter, she recurred twice during the MPA treatment and received debulking surgery each time. MPA was discontinued at age of 53, because another recurrent tumor grew up to 13 cm in diameter. Aromatase inhibitor anastrozole was then given at a daily dose of 1 mg with partial response (the tumor size decreased to 7 cm in diameter) for a duration of 9 months. After complete resection of the recurrent tumor, she remains progression-free for 16 months. Anastrozole was effective to recurrent low-grade ESS even after being refractory to progestin therapy. Aromatase inhibitor treatment may be a useful option as a second-line hormonal treatment to low-grade ESS.

K. Shoji · K. Oda (✉) · S. Nakagawa · K. Kawana · T. Yasugi · Y. Ikeda · S. Kozuma · Y. Taketani
Department of Obstetrics and Gynecology, Faculty of Medicine, The University of Tokyo, 7-3-1 Hongo Bunkyo-ku, Tokyo 113-8655, Japan
e-mail: katsutoshi-tky@umin.ac.jp

Y. Takazawa
Department of Pathology, Faculty of Medicine, The University of Tokyo, 7-3-1 Hongo Bunkyo-ku, Tokyo 113-8655, Japan

Published online: 31 March 2010

Keywords Low-grade endometrial stromal sarcoma · Uterine corpus · Recurrence · Aromatase inhibitor · Progestin therapy · Hormonal treatment

Introduction

Endometrial stromal sarcoma (ESS) is a rare neoplasm, accounting for 0.2% or less of gynecologic malignancies [1]. Low-grade ESS usually expresses estrogen receptors (ER) and progesterone receptors (PR), and estrogen acts as a growth stimulus [2, 3]. Objective responses have been obtained with progestin therapy, such as megestrol acetate and medroxyprogesterone acetate (MPA) [4, 5]. More recently, the efficacy of a non-steroid aromatase inhibitor has been also reported [6, 7], as it inhibits estrogen synthesis. Although either type of hormonal therapy might be useful as a first-line therapy, it is still uncertain whether a second-line hormonal treatment is effective to repetitively recurrent ESS with resistance to a first-line therapy.

We report a case of recurrent low-grade ESS with long-term survival, treated with MPA for 13 years as a first line and aromatase inhibitor anastrozole for 9 months as a second-line hormonal therapy.

Case report

A 34-year-old woman (gravida 4, para 2) underwent a total abdominal hysterectomy for presumed uterine fibroma at her local hospital in 1988. The histopathological result revealed stage I low-grade ESS of the corpus uteri. In December 1993, she was referred to our hospital, and a computed tomography (CT) scan revealed a 9-cm pelvic mass, bilateral ovarian masses (4 cm on the left and 7 cm

Humana Press

on the right), and para-aortic lymph node enlargement. She underwent secondary debulking surgery, including bilateral salpingo-oophorectomy, omentectomy, bowel resection, and biopsy of para-aortic lymph nodes. All the residual tumors were less than 1 cm in diameter. The final pathology revealed recurrence of the low-grade ESS (Fig. 1a), involving the bilateral adnexae, ileum, appendix, colon, omentum, and para-aortic lymph nodes. Immunohistochemical analysis showed a strong nuclear staining for both ER and PR (Fig. 1b, c), as well as CD10 (Fig. 1d) and vimentin, and a negative staining for HHF35, 1A4, Desmin, and CD34. Postoperatively, she was started on MPA at a daily dose of 600 mg. Three years after the MPA therapy, complete response was pathologically confirmed by second look laparoscopy. MPA was continued at a daily dose of 200–400 mg without any appreciable adverse effects.

In April 2000, surgical biopsy of a 2-cm mass around the liver confirmed the recurrence of the disease on peritoneum. Two years later, she received another debulking procedure with partial liver resection for a 5-cm tumor and resection of another 5-cm pelvic tumor. After the surgery, she was hospitalized four times within 2 years due to grade 2 ileus. In June 2006, a CT scan showed a 5-cm solid mass in the left upper quadrant. The patient did not choose a debulking surgery and was kept treated with MPA at a daily dose of 200–400 mg. Eight months later, she was found to have progression of disease, represented by enlargement of the mass up to 13 cm in diameter and appearance of 4 cm mesenteric mass in the pelvis (Fig. 2a). Then, MPA treatment was discontinued, and anastrozole at a daily dose of 1 mg was started with an informed consent. After 9 months of the treatment, the tumor in the left upper quadrant was decreased to 7 cm in diameter and the mesenteric tumor was undetected (Fig. 2b). Anastrozole was discontinued because of arthritis with grade 2 joint-function disorder. Then, she underwent complete resection of the recurrent tumor. Pathological findings also revealed the significant effect of anastrozole. As shown in Fig. 3, the majority of the tumor cells was necrotic and replaced by numerous foamy histiocytes. The viable cells remained partly in the marginal lesion with expression of ER and PR. She recovered from the joint-function disorder shortly after the surgery and remains asymptomatic and progression-free for 16 months.

Discussion

ESS is subdivided histopathologically into low-grade and undifferentiated (or high-grade) forms depending on the morphology, number of mitoses, cellularity, and necrosis. The primary treatment for low-grade ESS is mainly surgery, including an abdominal hysterectomy with bilateral salpingo-oophorectomy. Adjuvant treatment, such as radiotherapy or chemotherapy, is not routinely recommended

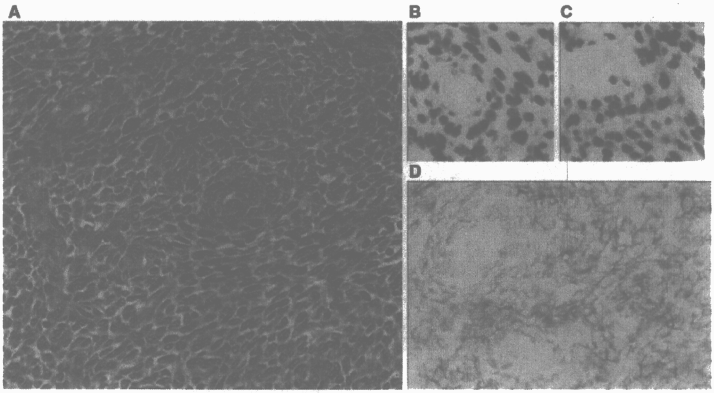

Fig. 1 Histological findings of the tumor, excised before MPA treatment. **a** High Power: Tumor cells in the pelvis, showing proliferation of endometrial stromal cells without significant atypia or pleomorphism, diagnosed as low-grade ESS. **b–d** High Power: Tumor cells are strongly positive for estrogen receptor (**b**) and progesterone receptor (**c**) and are diffusely positive for CD10 (**d**) by immunohistochemistry

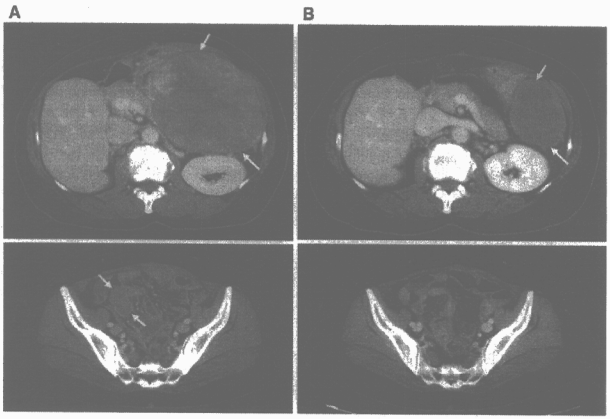

Fig. 2 Images of CT scan before and after anastrozole treatment. **a** Recurrent tumors with 13 cm in diameter in the left upper quadrant (*Upper*) and 4 cm in diameter in the pelvis (*Lower*). **b** The recurrent tumors were diminished to 7 cm in diameter (*Upper*) or became undetectable (*Lower*)

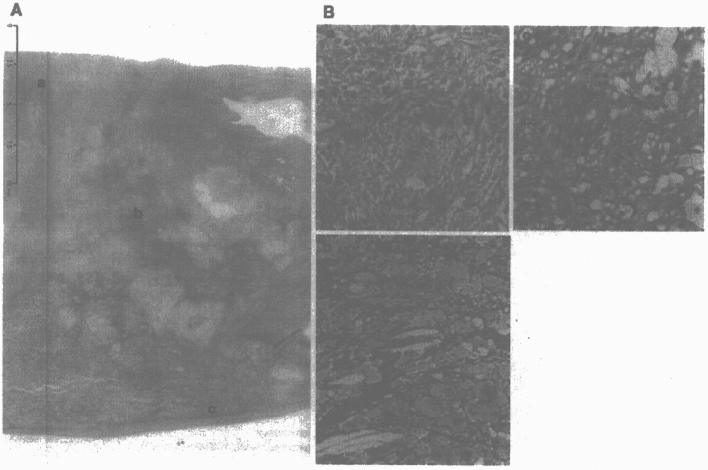

Fig. 3 Histological findings of the tumor, excised after anastrozole treatment. **a**. Low Power: Tumor cells with massive necrosis. **b** High Power: (*a*) Lesion with coagulative tumor cell necrosis, which occupies the majority of the tumor. (*b*) Center lesion with numerous foamy histiocytes. (*c*) Marginal lesion of the tumor with viable cells partly remaining

[8]. Although the prognosis of low-grade ESS is generally favorable with more than 90% of 5-year overall survival, the recurrence-free survival rate is reported to be about 50% [9, 10]. In addition to surgical resection, treatment option to recurrent low-grade ESS is hormonal therapy with progesterone derivative or aromatase inhibitor. MPA and megestrole acetate are synthetic derivatives of progesterone that exert an anti-estrogenic effect after binding to PR. The sensitivity to these progestin therapies is associated with the presence of ER and PR [11]. Aromatase inhibitors reduce estrogen levels by inhibiting its synthesis in peripheral sites. The distinct function suggests that suppressing aromatase might be still effective to recurrent ESS with resistance to progestin therapy.

The patient reported here suffered from repeated recurrences after becoming refractory to MPA treatment. Positive PR expression of the recurrent tumors suggests that the resistance to MPA therapy is caused by PR-independent manner. As a second-line hormonal therapy, anastrozole showed significant response to these recurrent tumors, suggesting that aromatase inhibitor might be useful for progestin-resistant low-grade ESS tumors. It is to be elucidated whether aromatase inhibitor is also effective to recurrent ESS tumors with negative PR expression.

Acknowledgments Conflict of interest statement All the authors declare no conflict of interest.

References

1. Koss LG, Spiro RH, Brunschwig A. Endometrial stromal sarcoma. Surg Gynecol Obstet. 1965;121:531–7.
2. Sabini G, Chumas JC, Mann WJ. Steroid hormone receptors in endometrial stromal sarcomas. A biochemical and immunohistochemical study. Am J Clin Pathol. 1992;97:381–6.
3. Wade K, Quinn MA, Hammond I, Williams K, Cauchi M. Uterine sarcoma: steroid receptors and response to hormonal therapy. Gynecol Oncol. 1990;39:364–7.
4. Gloor E, Schnyder P, Cikes M, Hofstetter J, Cordey R, Burnier F, et al. Endolymphatic stromal myosis. Surgical and hormonal treatment of extensive abdominal recurrence 20 years after hysterectomy. Cancer. 1982;50:1888–93.
5. Tsukamoto N, Kamura T, Matsukuma K, Imachi M, Uchino H, Saito T, et al. Endolymphatic stromal myosis: a case with positive estrogen and progesterone receptors and good response to progestins. Gynecol Oncol. 1985;20:120–8.
6. Maluf FC, Sabbatini P, Schwartz L, Xia J, Aghajanian C. Endometrial stromal sarcoma: objective response to letrozole. Gynecol Oncol. 2001;82:384–8.
7. Leunen M, Breugelmans M, De Sutter P, Bourgain C, Amy JJ. Low-grade endometrial stromal sarcoma treated with the aromatase inhibitor letrozole. Gynecol Oncol. 2004;95:769–71.
8. Reich O, Regauer S. Hormonal therapy of endometrial stromal sarcoma. Curr Opin Oncol. 2007;19:347–52.
9. Pink D, Lindner T, Mrozek A, Kretzschmar A, Thuss Patience PC, Dorken B, et al. Harm or benefit of hormonal treatment in metastatic low-grade endometrial stromal sarcoma: single center experience with 10 cases and review of the literature. Gynecol Oncol. 2006;101:464–9.
10. Kim WY, Lee JW, Choi CH, Kang H, Kim TJ, Kim BG, et al. Low-grade endometrial stromal sarcoma: a single center's experience with 22 cases. Int J Gynecol Cancer. 2008;18:1084–9.
11. Katz L, Merino MJ, Sakamoto H, Schwartz PE. Endometrial stromal sarcoma: a clinicopathologic study of 11 cases with determination of estrogen and progestin receptor levels in three tumors. Gynecol Oncol. 1987;26:87–97.

Immune Surveillance during Pregnancy

Shigeru Saito[1], Tomoko Shima[1], Akitoshi Nakashima[1], Yi Lin[2]

[1]Department of Obstetrics and Gynecology, University of Toyama, Japan. [2]Department of Obstetrics and Gynecology, School of Medicine, Institute of Obstetrics and Gynecology, Renji Hospital, Shanghai Jiantong University, Shanghai, China.

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

Address for correspondence : S. Saito, 2630 Sugitani Toyama 930-0194, Japan; Ph. No.: +81-76-434-7355; E-mail: s30saito@med.u-toyama.ac.jp

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

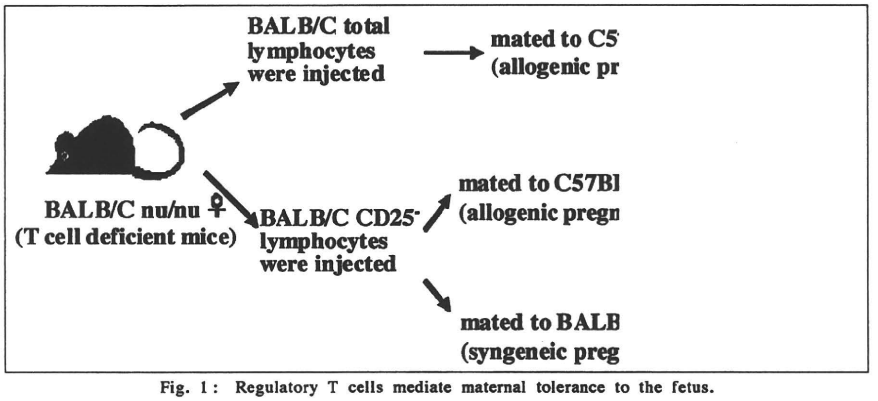

Fig. 1: Regulatory T cells mediate maternal tolerance to the fetus.

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.

References

1. Blois SM, Alba Soto CD, Tometten M, Klapp BF, Margni RA, Arck PC. Lineage, maturity, and phenotype of uterin murine dendritic cells throughout gestation indicate a protective role in maintaining pregnancy. *Biol Reprod* 2004; 70: 1018–1023.
2. Krey G, Frank P, Shaikly V, Barrientos G, Cordo-Russo R, Ringel F, et al. In vivo dendritic depletion reduces breeding efficiency, affecting implantation and early placental development in mice. *J Mol Med* 2008; 86: 999–1011.
3. Plaks V, Birnberg T, Berkutzki T, Sela S, BenYashar A, Kalchenko V, et al. Uterine DCs are crucial for decidua formation during embryo implantation in mice. *J. Clin Invest* 2008; 118: 3954–3965.
4. Kammerer U, Schoppet M, McLellan AD, Kapp M, Hupperts HI, Kampgen E, et al. Human decidua contains potent immunostimulatory CD83(+) dendritic cells. *Am J Pathol* 2000; 157: 159–169.
5. Breburda EE, Dambaeva SV, Slukvin II, Golos TG. Selective distribution and pregnancy-specific expression of DC-SIGN at the maternal-fetal interface in the rhesus macaque: DC-SIGN is a putative marker of the recognition of pregnancy. *Placenta* 2006; 27: 11–21.
6. Sakaguchi S. Regulatory T cells; key controllers of immunologic self-tolerance. *Cell* 2000; 101: 455–458.
7. Aluvihare VR, Kallikikourdis M, Betz AG. Regulatory T cells mediate maternal tolerance to the fetus. *Nat Immunol* 2004; 5: 266–271.
8. Zenclussen AC, Gerlof K, Zenclussen ML, Sollwedel A, Bertoja AZ, Ritter T, et al. 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* 2005; 166: 811–122.
9. 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.
10. 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.
11. Robertson SA, Guerin LR, Bromfield JJ, Branson KM, Ahlstrom AC, Care AS. Seminal fluid drives expansion of the $CD4^+$ $CD25^+$ T regulatory cell pool and induces tolerance to paternal alloantigens in mice. *Biol Reprod* 2009; 80: 1036–1045.
12. Ebata K, Shimizu Y, Nakayama Y, Minemura J, Kato T., Yasumura S, et al. Immature natural killer cells suppress dendritic cell functions during the development of leukemia in a mouse model. *J Immunol* 2006; 176: 4113–4124.
13. Lin Y, Zhong Y, Saito S, Chen Y, Shen W, Di J, et al. Characterization of natural killer cells in nonobese diabetic/severely compromised immunodeficient mice during pregnancy. *Fertil Steril* 2009; 91: 2676–2686.
14. Nishikawa K, Saito S, Morii T, Hamada K, Ako H, Narita H, et al. Accumulation of $CD16^-$ $CD56^+$ natural killer cells with high affinity interleukin 2 receptors in human early pregnancy decidua. *Int Immunol* 1991; 3: 743–750.
15. Umekage H, Saito S, Morikawa H. Enhancement by stem cell factor of interleukin-2 (IL-2)-induced DNA synthesis in human decidual $CD16^-CD56^{bright}$ natural killer cells mediated by increased expression of the IL-2 receptor ± chain. *J Reprod Immunol* 1998; 40: 1–24.

Th1/Th2/Th17 and Regulatory T-Cell Paradigm in Pregnancy

Shigeru Saito, Akitoshi Nakashima, Tomoko Shima, Mika Ito

Department of Obstetrics and Gynecology, University of Toyama, Toyama, Japan

Keywords
Abortion, cytokine network, implantation, preterm labor, reproduction

Correspondence
Shigeru Saito, MD, PhD, Department of Obstetrics and Gynecology, University of Toyama, 2630 Sugitani, Toyama-shi, Toyama 930-0194, Japan.
E-mail: s30saito@med.u-toyama.ac.jp

Submitted March 5, 2010;
accepted March 5, 2010.

Citation
Saito S, Nakashima A, Shima T, Ito M. Th1/Th2/Th17 and regulatory T-cell paradigm in pregnancy. Am J Reprod Immunol 2010; 63: 601–610

doi:10.1111/j.1600-0897.2010.00852.x

T-helper (Th) cells play a central role in modulating immune responses. The Th1/Th2 paradigm has now developed into the new Th1/Th2/Th17 paradigm. In addition to effector cells, Th cells are regulated by regulatory T (Treg) cells. Their capacity to produce cytokines is suppressed by immunoregulatory cytokines such as transforming growth factor (TGF)-β and interleukin (IL)-10 or by cell-to-cell interaction. Here, we will review the immunological environment in normal pregnancy and complicated pregnancy, such as implantation failure, abortion, preterm labor, and preeclampsia from the viewpoint of the new Th1/Th2/Th17 and Treg paradigms.

Introduction

T cells play a central role in immunoregulation and immunostimulation. T-helper (Th) cells can be classified into Th1 cells, which produce interleukin (IL)-2 and interferon (IFN) γ and are involved in cellular immunity, and Th2 cells, which produce IL-4, IL-5 and IL-13 and are involved in humoral immunity.[1] In the 1980s to 1990s, maternal tolerance toward fetal alloantigens was explained by the predominant Th2-type immunity during pregnancy, which overrules Th1-type immunity, therefore protecting the fetus from maternal Th1-cell attack.[2] Indeed, predominant Th1-type immunity has been observed in recurrent spontaneous abortion[3,4] and in preeclampsia.[5] However, Th2-dominant immunity was also reported in recurrent abortion cases,[6,7] and therefore, the Th1/Th2 paradigm is now insufficient to explain the mechanism of why the fetus is not rejected by maternal immune cells. Now, the Th1/Th2 paradigm has been expanded into the Th1/Th2/Th17 and regulatory T (Treg) cells paradigm.[8] Th17 cells, which produce the proinflammatory cytokine, IL-17, play important roles for the induction of inflammation.[8,9] They have been proposed as a pathogenetic mechanism in autoimmune diseases and acute transplant rejection. In contrast, Treg cells play central roles for immunoregulation and induction of tolerance. Treg cells are now known to inhibit proliferation and cytokine production in both $CD4^+$ and $CD8^+$ T cells, immunoglobulin production by B cells, cytotoxic activity of natural killer (NK) cells, and maturation of dendritic cells (DCs), resulting in induction of tolerance.[10,11]

This review aims to reexamine the Th1/Th2/Th17 and Treg paradigms in normal pregnancy and complicated cases such as implantation failure, recurrent pregnancy loss, and preterm labor.

Reciprocal developmental pathways between Th1/Th17 subsets and between Th17/Treg subsets

Th1 cells are characterized by the transcription factor T-bet and signal transducer and activator of

© 2010 John Wiley & Sons A/S

transcription (STAT) 4, and the production of IL-2, IFN-γ, and tumor necrosis factor (TNF) β. They are involved in the cellular immunity and rejection process. In contrast, mediators of humoral immunity, Th2 cells develop into IL-4-, IL-5-, and IL-13-producing cells by the transcription factor GATA-3 and STAT6. A new lineage of Th cells that selectively produce IL-17 has been proposed. This population, which has been termed Th17, plays a critical role for the induction of inflammation and plays a critical role in the pathogenesis of autoimmune diseases and rejection.[8,9] Th17 cells are characterized by unique signaling pathways, receptor-related orphan receptor (ROR) C2 or ROR-α. Th17 cells are involved in the host defense against bacteria, fungi, and viruses. But evidence for the pathogenic role of Th17 in rheumatoid arthritis (RA), psoriasis, multiple sclerosis, and inflammatory bowel disease has been reported.[8,9]

The function of effector T cells, such as Th1, Th2 and Th17 cells, is regulated by $CD4^+$ $CD25^+$ regulatory T (Treg) cells. $CD4^+$ $CD25^+$ Treg cells are important cells for the maintenance of peripheral tolerance.[10,11] The master gene for the differentiation to Treg cells is transcription factor forkhead box P3 (Foxp3).

Interestingly, recent data show the reciprocal development pathways between Th17/Th1 subset and Th17/Treg subsets[8] (Fig. 1). The progenitor cells differentiate to Th17/Treg intermediate cells, which express both RORC and Foxp3. The differentiation of both Th17 and Treg cells requires transforming growth factor (TGF)-β. When DCs are activated by microorganisms that induce production of pro-inflammatory cytokine IL-6 or IL-1, TGF-β induced differentiation of native T cells diverted away from the induced Treg cells pathway and toward the Th17 cells pathway. The differentiation of human Th17 cells is inhibited by high TGF-β concentrations but requires IL-1β and IL-6.[8,9] Prostaglandin E_2 (PGE_2), which is a mediator of tissue inflammation, induces up-regulation of IL-23R and IL-1R and promotes the differentiation and expansion of Th17 cells. The plasticity of Th17/Th1 lineage has also been reported.[8] The existence of $IFNγ^+$ $IL\text{-}17^+$-double positive T cells has been reported. Stimulation by IL-12 down-regulates IL-17 production and induces IFNγ production, suggesting Th1 polarization (Fig. 1), but interplay between the Th1 and Th17 lineages has not been fully clarified.

Interestingly, the conversion of Treg cells to Th17 cells has been reported in humans and mice. IL-6

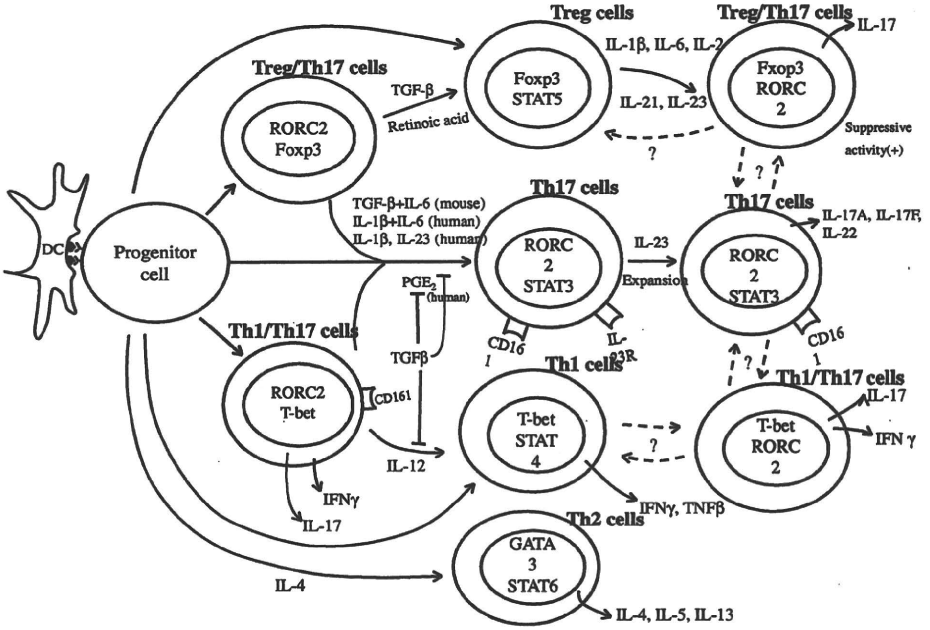

Fig. 1 Th1, Th2, Th17, and Treg cells development from $CD4^+$ progenitors. Plasticity of T-cell phenotype between Th1 cells and Th17 cells or Treg cells and Th17 cells is reported.

© 2010 John Wiley & Sons A/S

plays an important role in this process in mice, and IL-1β and IL-2 are the key cytokines in this process in humans.

These data show that Th1/Th2/Th17 and Treg lineages are associated with each other, and they are able to convert to other lineages.

Th1/Th2/Th17 and Treg paradigms in implantation

Studies in humans and mice have shown that leukemia inhibitory factor (LIF), IL-1, IL-6, IL-11, vascular endothelial growth factor, placenta growth factor, fibroblast growth factor-2, heparin-binding epidermal growth factor (HB-EGF), and TGF-β play important roles for successful implantation by modulating angiogenesis processes, trophoblast differentiation, or the immune system.[12] Recent data show that uterine DCs play a central role for successful implantation.[13,14] The number of uterine DCs increases at the implantation period, and depletion of DCs results in severe implantation failure.[13,14] DC depletion impairs uterine NK cell maturation, tissue remodeling, and angiogenesis.[13,14] Surprisingly, depletion of DCs also causes embryo resorption in syngeneic and T-cell-deficient pregnancy, suggesting that DCs appear to govern uterine receptivity independent of the immunological tolerance.[14] Although tolerogenic DCs take a part in inducing maternofetal tolerance, DCs may play a principal role in implantation. But depletion of Treg cell by anti-CD25 monoclonal antibody on the day or 2.5 days after mating results in severe impairment of implantation in allogeneic mice, but this was not observed in syngeneic mice, suggesting that Treg cells are essential for inducing immunological tolerance.[15,16] Treg cells are already increased in the lymph nodes draining the uterus 2 or 3 days after mating, suggesting that Treg cells accumulate into the lymph nodes draining the uterus before the implantation.[13,14] Robertson et al. reported seminal plasma, but not sperm, plays an essential role for the induction of paternal antigen-specific tolerance.[19] The maternal immune system prepares for the semiallograftic embryo to come into the uterus. Alvihare et al. reported that expansion of Treg cells in the lymph nodes draining uterus was also observed in allogeneic mice and syngeneic mice.[17] They proposed that pregnancy hormones such as estrogen might induce Treg cells numbers and Arruvito et al.[18] showed that Treg cells increased during the follicular phase of the menstrual cycle, suggesting that estrogen plays a part for the expansion of Treg. But our recent data showed paternal antigen-specific Treg cells proliferate in the lymph nodes draining the uterus 3 days after coitus. When BALB/c female mice were mated to DBA/2 male mice, fetuses express DBA/2-derived Mls Ia antigen on the cell surface. As Mls Ia antigen is recognized by T-cell receptor Vβ6, $CD4^+$ $CD25^+$ $Foxp3^+$ $V\beta6^+$ cells are paternal antigen-specific Treg cells. Ki67 is a marker for cell-proliferation; therefore, $Ki67^+$ $CD4^+$ $CD25^+$ - $Foxp3^+$ $V\beta6^+$ cells are proliferating fetal antigen-specific Treg cells. Interestingly, $Ki67^+$ $V\beta6^+$ Treg cells increase in the uterine draining lymph node on 3.5 days post-coitus in BALB/c x DBA/2 mating but not in BALB/c x BALB/c mating, suggesting that paternal antigen-specific Treg cells proliferate in the uterine draining lymph node before the implantation.[19] After implantation, on day 5.5 post-coitus, $Ki67^+$ paternal antigen-specific Treg cells increase in the uterus in BALB/c × DBA/2 mating.[20] These findings demonstrate that paternal antigen-specific Treg cell in draining lymph nodes quickly move to the pregnant uterus and proliferate in the uterus, resulting in the induction of paternal antigen-specific tolerance at the early stage of pregnancy (Fig. 2).[20] The ligands such as CCR2, CCR4, CCR5, CCR6, and CD62L are known as molecules for Treg cell migration. CCL2, CCL22, CCL17, CCL4, CCL20, or L-selection in the uterus might selectively accumulate Treg cells from peripheral tissues.[21] Recent data demonstrate that some Treg cells express LH/CG receptor, and human chorionic gonadotropin produced by human trophoblast has an ability to attract Treg cells to the uterus.[22] Further studies are needed to clarify whether paternal antigen-specific Treg cells express these chemokine receptors or LH/CG receptors on their surface. Th1/Th2/Th17 balance at implantation in such Treg cell-deficient or depleted mice has not been reported, and further studies are needed.

In humans, augmented Th1-type immunity or suppressed Th1-type immunity in endometrium is observed in repeated implantation failure.[23] Deceased number or function of Treg cells might be a cause of augmented Th1-type immunity in such cases. Jasper et al.[24] reported that primary unexplained infertility is associated with reduced expression of Foxp3 mRNA in endometrial tissue. It can be speculated that decreased Treg cells might induce implantation failure, resulting in unexplained infertility.

© 2010 John Wiley & Sons A/S

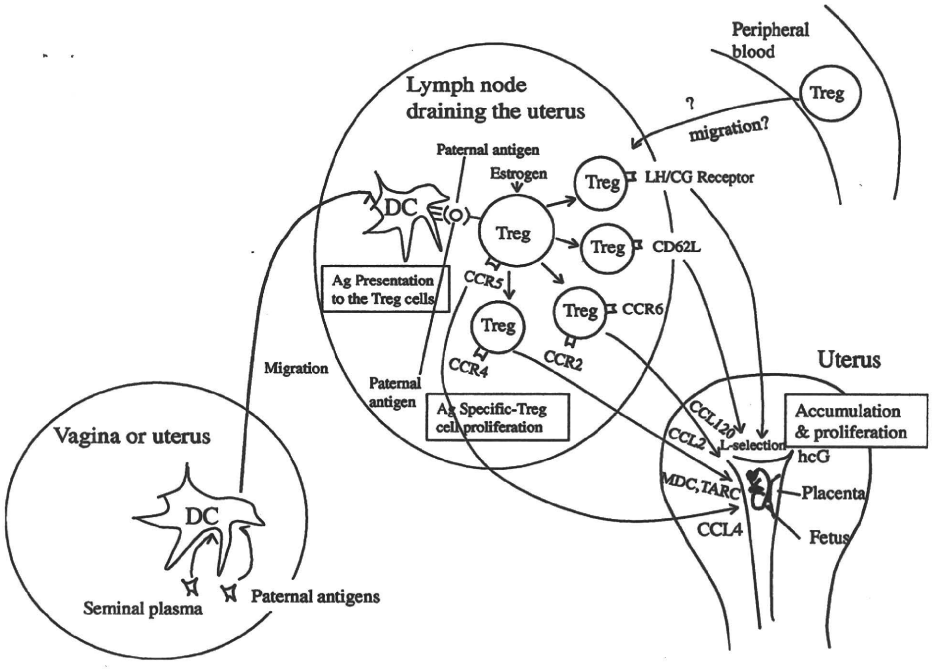

Fig. 2 A model for the paternal antigens-specific Treg cells expansion, proliferation, and mobilization from vagina to pregnant uterus. As a first step, DCs uptake paternal antigen, and these DCs migrate to lymph nodes draining the uterus. DCs present paternal antigens to Treg cells, and Treg cells proliferate before the implantation. These paternal antigen-specific Treg cells migrate to the pregnant uterus by chemokine and hcG-induced chemoattractant mechanisms.

Th1/Th2/Th17 and Treg paradigms in normal pregnancy

Many studies have reported a predominant Th2-type immunity and suppressed Th1-type immunity during pregnancy.[2,3,25] This tendency is more clear at the feto–maternal interface (Table 1). Both Th2 cells and T cytotoxic (Tc) 2 cells accumulate in the decidua basalis,[26,27] and uterine DC cells can differentiate naïve T cells to Th2 cells.[28] Therefore, both Th2 cell migration and Th2 cell differentiation induce Th2-type immunity at the feto–maternal interface. But the systemic immune system does not change so much.[25,26] Moreover, IL-4, IL-5, IL-9, and IL-13 knockout mice show normal pregnancy in allogeneic pregnancy,[28] suggesting that predominant Th2-type immunity might not be essential for successful pregnancy. Administration of excess amount of Th1-type cytokine such as IL-2 or IFNγ induces abortion in mice, and stimulation of toll-like receptor (TLR) induces Th1-type cytokine production, resulting in abortion.[29] But IFNγ also plays an important role for vascular remodeling at the early stage of murine pregnancy.[30] Thus, Th1-type immunity is well controlled to avoid overstimulation of Th1-type immunity. Treg cells might play a part in this process.

IL-17 plays an important role for the pathophysiology in RA.[8,9] The symptoms of RA usually improve during pregnancy,[2] suggesting that Th17 cells might be decreased during pregnancy (Table 1).

Two recent articles show that the frequency of circulating Th17 cells to $CD4^+$ T cells is very low (0.64–1.4%) in healthy subjects.[31,32] In our study, the frequency of Th17 cells to $CD4^+$ T cells during all stages of pregnancy period was similar to that in non-pregnant women,[32] but Santner-Nanan et al.[33] reported that the frequency of Th17 cells in the third trimester of pregnancy was significantly lower compared to that of non-pregnant women. Further studies are needed to determine circulating Th17 cell levels during pregnancy. The main producer of IL-17 in both peripheral blood and deciduas is $CD4^+$ T cells, and IL-17-expressing $CD8^+$ T cells are rare (approximately 0.1%).[32] $CD14^+$ monocytes,

American Journal of Reproductive Immunology 63 (2010) 601–610
© 2010 John Wiley & Sons A/S

$CD56^{dim}$ NK cells, and $CD56^{bright}$ NK cells do not express IL-17.[34]

Previous studies demonstrate that elevation of IL-17 is observed in acute renal rejection,[35] suggesting that increased Th17 cells in pregnancy decidua might be disadvantageous for the maintenance of pregnancy.[33]

Unexpectedly, the frequency of Th17 cells in the decidua is significantly higher compared to that in peripheral blood. The uterine cavity is not completely sterile, and therefore, Th17 cells might play a role to induce protective immune response against extracellular microbes. Recent reports show that IL-17 increases progesterone secretion by JEG-3 human choriocarcinoma cells and induces the invasive capacity of JEG cells.[36] These data suggest IL-17 may be useful for a successful pregnancy.

Inflammation is necessary for successful implantation, but excessive inflammation can cause embryo resorption. Treg cells might regulate excessive inflammation in the uterus at the implantation period. Indeed, endometrial (decidual) Treg cells increase in mice and humans,[16,18,37–39] and Treg cell number in allogeneic pregnant mice is much higher compared to that in syngeneic pregnancy (Table 1).[39] And these Treg cells in the decidua selectively inhibit the mixed lymphocytes reaction (MLR) to umbilical mononuclear cells, suggesting selective migration of fetal antigen-specific Treg cells to the decidua.[40] In humans, extravillous trophoblasts express polymorphic histocompatibility antigen, HLA-C, which can elicit an allogeneic T-cell-response. Tilburgs et al.[41] recently reported that pregnancy with a HLA-C-mismatched child induces an increased percentage of activated T cells in decidual tissue. Interestingly, HLA-C-mismatched pregnancies exhibit significantly increased suppressive capacity in one-way MLR reaction to umbilical mononuclear cells, suggesting decidual Treg cells inhibit HLA-C-recognized-T-cell attack. These findings show that Treg cells also play an important role for regulating fetal rejection by maternal immune cells in human pregnancy.

Th1/Th2/Th17 and Treg paradigms in abortion

Predominant Th1-type immunity is observed in abortion, but predominant Th2-type immunity is also reported in recurrent pregnancy loss.[2–4,6,7] Therefore, adequate balance for Th1/Th2 immunity, i.e., slightly shifted to Th2-type immunity, may be suitable for the maintenance of pregnancy. Overstimulation of Th1 immunity or Th2 immunity might be harmful for successful pregnancy. A pro-inflammatory cytokine IL-17 induces the expression of many mediators of inflammation. Recent data show an increased prevalence of Th17 cells in peripheral blood and decidua in unexplained recurrent spontaneous abortion patients.[42,43] The master transcription factor for Th17 cells, RORc and IL-23, which play a crucial role in Th17 cells expansion, is also increased in the decidual tissue in recurrent spontaneous abortion cases.[43] An inverse relationship between the numbers of Th17 cells and Treg cells is observed in peripheral blood and decidua of unexplained recurrent spontaneous abortion cases.[43] IL-6 is a key cytokine that blocks the development of Treg cells and induces the differentiation of Th17 cells. The serum IL-6 and soluble IL-6 receptor (sIL-6R) levels are increased. On the other hand, the trans-signaling inhibitor, soluble gp130 (sgp130) level, is decreased in recurrent spontaneous cases.[44] After paternal lymphocytes alloimmunization, sIL-6R levels are decreased and sgp130 levels are increased, and Treg cell number is increased.[44] These findings suggest IL-6 trans-signaling plays a very important role for regulating Th17/Treg balance.

We have already reported that Th17 cells increased in decidua of inevitable abortion, i.e., progression stage of abortion.[42] And the number of IL-17^+ cells is well correlated with that of neutrophils, suggesting that IL-17 plays an important role in neutrophil infiltration. But Th17 cells did not change in the decidua of missed abortion cases that did not show vaginal bleeding, uterine cramping or cervical deletion.[42] Importantly, all the abortion cases in Wang's study were inevitable abortion.[43] These findings suggest that increased Th17 cells are a consequence of fetal loss, but not a cause of fetal loss. After embryonic death, increased IL-1 or IL-6 production and decreased TGF-β production might cause increased Th17 cells and decreased Treg cells in the uterus.

Our group first reported the decreased numbers of peripheral and decidual Treg cells in spontaneous abortion cases.[37] Yang et al.[45] reported that the proportion of Treg cells in both decidua and peripheral blood in unexplained recurrent spontaneous abortion was significantly lower than those in control women. And the immunosuppressive activity of Treg cells in repeated miscarriage cases have reduced

© 2010 John Wiley & Sons A/S

Table 1 Th1/Th2/Th17 and Treg Cells in Normal Pregnancy and Abortion

	Normal pregnancy		Abortion		
	Peripheral blood	Uterus	Peripheral blood	Uterus	Depletion of Th1, Th2,Th17 or Treg cells
Th1 cells	↘	↓	↗ →	↑ →	Abortion is not observed.
Th2 cells	↗	↑	→	↓ → ↑(conflict data)	Abortion is not observed.
Th17 cells	→ ↘	↗	→ ↗	→ (missed abortion) ↑ (inevitable abortion) ↑ (recurrent abortion: inevitable abortion)	There is no data, but IL-17 null mice are fertile.
Treg cells	↑	↑↑	→	→	Abortion and implantation failure are observed in allogeneic pregnancy.

→ : no change, ↗: slightly elevate, ↑: elevate, ↑↑: markedly elevate, ↘: slightly decrease, ↓: decrease.

suppressive capacity compared with normal fertile women.[18] Persistent TLR stimulation or IL-6 suppression of Treg function have been reported,[46,47] and therefore these reduced Treg functions in recurrent spontaneous abortion might be caused by chronic inflammation. In a mouse model, when BALB/c $CD25^-$ lymphocytes are injected into BALB/c nu/nu T-cell-deficient mice, they did not have $CD4^+$ $CD25^+$ Treg cells. These Treg-deficient mice showed abortion in allogeneic pregnancy, but this was not observed in syngeneic pregnancy.[17] But it has not been clarified how greatly Treg cells induce abortion in allogeneic pregnancy. We have injected various amounts of anti-CD25 monoclonal antibody on 4.5 and 7.5 days after mating and found that an over 60% decrease of Treg cells could induce abortion in allogeneic pregnancy.[48] CBA/J female mice mated with DBA/2J male mice is a good model for abortion. Interestingly, Zenclussens et al.[16] reported fewer Treg cells associated with elevated Th1-type immunity in CBA/J × DBA/2J mating. Adoptive transfer of $CD4^+$ $CD25^+$ cells from normal pregnant mice prevented fetal loss, but adoptive transfer of $CD4^+$ $CD25^+$ T cells from non-pregnant mice had no effect for protecting fetal loss. Transfer of $CD4^+$ $CD25^+$ T cells from pregnant mice on day 4 of pregnancy did not prevent abortion.[16] Interestingly, transfer of $CD4^+$ $CD25^+$ Treg cells to abortion-prone mice induced the expression of LIF and TGF-β, but the expression of Th1-type cytokines such as IFNγ and TNFαwas unchanged, and Th1/Th2 ratio did not change.[49] These findings show that the protective effect against abortion by Treg cells might not be caused by the correction of abnormal Th1/Th2 balance.

Th1/Th2/Th17 and Treg paradigms in preterm labor

Preterm labor is associated with subclinical infection, and intrauterine inflammatory response causes preterm labor and delivery. Therefore, the secretion of pro-inflammatory cytokines such as IL-1, IL-6, IL-8, and TNFα is increased in amniotic fluid, decidual tissue, and chronic tissue. Although the secretion of Th1-type cytokines, i.e., IL-2, IFNγ, and TNFα, which mediate inflammatory reactions, is up-regulated in preterm labor,[50] the secretion of Th2-type cytokines, i.e., IL-4 and IL-6, is also elevated.[51]

TGF-β might be a key cytokine that regulates Th17/Treg lineage. As IL-17 is a key cytokine that induces inflammation, IL-17 might have some roles in pathophysiology of preterm labor. Our groups recently found that Th17 cell number is increased in the chorioamniotic membrane of preterm delivery cases with chorioamnionitis (CAM).[34] Amniotic fluid IL-17 levels in severe CAM (stage III) preterm delivery cases were significantly higher than those in CAM-negative preterm delivery cases. Amniotic fluid IL-17 levels were positively correlated with IL-8 levels. Interestingly, although IL-17 did not enhance IL-8 secretion by amniotic mesenchymal cells, TNFα-induced IL-8 secretion was enhanced by IL-17 in a dose-dependent manner. Amniotic mesenchymal cells express IL-17 receptor, and therefore, IL-17 signaling pathway might up-regulate TNFα-induced IL-8 secretion by amniotic mesenchymal cells. Indeed, the IKK inhibitor and MAPK inhibitor significantly inhibited IL-17 with TNFα-induced IL-8 secretion in amniotic mesenchymal cells.[34] These findings show that Th17 cells promote inflammation at the feto–maternal interface in preterm delivery.

© 2010 John Wiley & Sons A/S

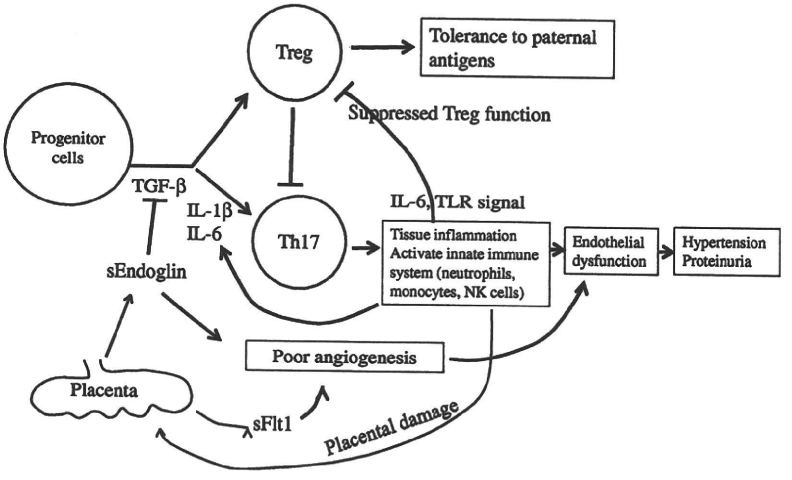

Fig. 3 The interrelationship between inflammation, poor angiogenesis, endothelial damage, and imbalance of Th17 and Treg differentiation.

Th1/Th2/Th17 and Treg paradigms in preeclampsia

In the early days of reproductive immunology when the Th1/Th2 paradigm was first proposed, Th1/Th2 balance in preeclampsia was examined by measuring cytokines or detecting intracellular cytokines by flow cytometry.[5] It has been clarified that predominant Th1-type immunity is present in preeclampsia.[5] A later study demonstrated that the production of Th1-type cytokine, IFN-γ, is also enhanced in NK cells.[52] Secreted IL-12p70 and IL-18, which differentiate Th1 cells from the progenitor cells, are increased in preeclampsia.[5,52] Predominant Th1-type immunity may suppress the tolerance system, resulting in preeclampsia.

As a next stage, our interest shifted to the Treg cells in preeclampsia, because Treg cells were proposed as major contributors to the maintenance of tolerance during pregnancy.[17,37,38] Recent reports described decreased numbers of Treg cells in preeclampsia,[53,54] although two other articles found stable Treg cells in preeclampsia.[55,56] However, the sample number was too small in one study,[56] and the other study[55] evaluated only total $CD4^+$ $CD25^+$ as Treg cells. The frequency of a $CD4^+$ $CD25^{high}$ cell population was not described. In humans, Treg cells are $CD4^+$ $CD25^{high}$ cells, and $CD4^+$ $CD25^{dim}$ cells have no ability for immunoregulation.

Recently, Santner-Nanan et al.[33] reported very interesting findings. They studied the frequency of $CD4^+$ $CD25^{high}$, $CD4^+$ $CD127^{low}CD25^+$, and $CD4^+$ $Foxp3^+$ cells in preeclampsia. Treg cells are involved in all of these three populations. The frequencies of the three populations in preeclampsia were significantly lower than those in normal pregnancy, and the immunosuppressive function of Treg cells did not change in preeclampsia. These findings strongly support that inadequate tolerance because of small numbers of Treg cells may be present in preeclampsia.

Recently, the developmental and functional links between induced Treg (iTreg) cells and Th17 cells have been reported.[8,9] Th17 cells and iTreg cells share a requirement for TGF-β, and high TGF-β concentrations induce Treg cells. Terminal differentiation of Th17 cells requires IL-1β and TGF-β.[57] An imbalance between Treg cells and Th17 cells has been proposed as a pathogenic mechanism in several human diseases. Santner-Nanan et al. recently reported an increased population of peripheral blood Treg cells and a decreased population of peripheral Th17 cells in normal pregnancy. In contrast, a decreased population of Treg cells and an increased population of Th17 cells compared to non-pregnancy levels in preeclampsia have been reported.[33]

Preeclampsia is associated with exaggerated systemic inflammatory changes[58] and poor angiogenesis because of increased levels of soluble Flt-1 and soluble endoglin[59] (Fig. 3). Very interestingly, soluble endoglin is an inhibitor of TGF-β; therefore, TGF-β signaling is suppressed in preeclampsia. Furthermore, IL-1β and IL-6, which induce Th17 cell differentiation, are produced by monocytes in preeclampsia. These factors may easily differentiate Th17 cells, which induce exaggerated inflammation, resulting decreased numbers of Treg cells. Increased Th17 cells

© 2010 John Wiley & Sons A/S

may induce exaggerated systemic inflammatory changes and vascular endothelial dysfunction. Furthermore, chronic inflammation may impair Treg function.[47] The chronic inflammation theory, endothelial dysfunction theory, poor angiogenesis theory, and immune maladaption theory are interrelated, and the imbalanced differentiation of Treg cells and Th17 cells may explain the pathophysiology of preeclampsia.

Conclusions

The new paradigm, i.e., the Th1/Th2/Th17 and Treg paradigms, has been proposed, and emerging literature provides many new findings in reproductive biology and immunology. The balance and correlation between Th1 cells, Th2 cells, Th17 cells, and Treg cells should be discussed to understand reproductive immunology, and it is important to find new therapies for implantation failure, recurrent pregnancy loss, preterm labor, and preeclampsia.

References

1 Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, Coffman RL: Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J Immunol* 1986; 136:2348–2357.

2 Wegmann TG, Lin H, Guilbert L, Mosmann TR: Bidirectional cytokine interactions in the maternal-fetal relationship: is successful pregnancy a TH2 phenomenon? *Immunol Today* 1993; 14:353–356.

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 Raghupathy R: Th1-type immunity is incompatible with successful pregnancy. *Immunol Today* 1997; 18:478–482.

5 Saito S, Sakai M: Th1/Th2 balance in preeclampsia. *J Reprod Immunol* 2003; 59:161–173.

6 Chaouat G, Lédée-Bataille N, Zourbas S, Ostojic S, Dubanchet S, Martal J, Frydman R: Cytokines, implantation and early abortion: re-examining the Th1/Th2 paradigm leads to question the single pathway, single therapy concept. *Am J Reprod Immunol* 2003; 50:177–186.

7 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.

8 Peck A, Mellins ED: Plasticity of T-cell phenotype and function: the T helper type 17 example. *Immunology* 2009; 129:147–153.

9 Crome SQ, Wang AY, Levings MK: Translational mini-review series on Th17 cells: function and regulation of human T helper 17 cells in health and disease. *Clin Exp Immunol* 2010; 159:109–119.

10 Sakaguchi S: Naturally arising Foxp3-expressing CD25 + CD4 + regulatory T cells in immunological tolerance to self and non-self. *Nat Immunol* 2005; 6:345–352.

11 Akbar AN, Vukmanovic-Stejic M, Taams LS, Macallan DC: The dynamic co-evolution of memory and regulatory CD4+ T cells in the periphery. *Nat Rev Immunol* 2007; 7:231–237.

12 Yoshinaga K: Review of factors essential for blastocyst implantation for their modulating effects on the maternal immune system. *Semin Cell Dev Biol* 2008; 19:161–169.

13 Krey G, Frank P, Shaikly V, Barrientos G, Cordo-Russo R, Ringel F, Moschansky P, Chernukhin IV, Metodiev M, Fernández N, Klapp BF, Arck PC, Blois SM: In vivo dendritic cell depletion reduces breeding efficiency, affecting implantation and early placental development in mice. *J.Mol Med* 2008; 86:999–1011.

14 Plaks V, Birnberg T, Berkutzki T, Sela S, BenYashar A, Kalchenko V, Mor G, Keshet E, Dekel N, Neeman M, Jung S: Uterine DCs are crucial for decidua formation during embryo implantation in mice. *J Clin Invest* 2008; 118:3954–3965.

15 Darrasse-Jèze G, Klatzmann D, Charlotte F, Salomon BL, Cohen JL: CD4+CD25+ regulatory/suppressor T cells prevent allogeneic fetus rejection in mice. *Immunol Lett* 2006; 102:106–109.

16 Zenclussen AC, Gerlof K, Zenclussen ML, Sollwedel A, Bertoja AZ, Ritter T, Kotsch K, Leber J, Volk HD: 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* 2005; 166:811–822.

17 Aluvihare VR, Kallikourdis M, Betz AG: Regulatory T cells mediate maternal tolerance to the fetus. *Nat Immunol* 2004; 5:266–271.

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

© 2010 John Wiley & Sons A/S

19 Robertson SA, Guerin LR, Bromfield JJ, Branson KM, Ahlström AC, Care AS: Seminal fluid drives expansion of the CD4+CD25+ T regulatory cell pool and induces tolerance to paternal alloantigens in mice. *Biol Reprod* 2009; 80:1036–1045.
20 Shima T: Paternal antigen-specific regulatory T cells proliferate in the draining lymph nodes before the implantation and proliferate in the uterus after the implantation, and are necessary for maintenance of allogeneic pregnancy 2010 ASRI Annual Meeting Abstract.
21 Guerin LR, Prins JR, Robertson SA: Regulatory T-cells and immune tolerance in pregnancy: a new target for infertility treatment? *Hum Reprod Update* 2009; 15:517–535.
22 Schumacher A, Brachwitz N, Sohr S, Engeland K, Langwisch S, Dolaptchieva M, Alexander T, Taran A, Malfertheiner SF, Costa SD, Zimmermann G, Nitschke C, Volk HD, Alexander H, Gunzer M, Zenclussen AC: Human chorionic gonadotropin attracts regulatory T cells into the fetal-maternal interface during early human pregnancy. *J Immunol* 2009; 182:5488–5497.
23 Lédée-Bataille N, Dubanchet S, Coulomb-L'hermine A, Durand-Gasselin I, Frydman R, Chaouat G: A new role for natural killer cells, interleukin (IL)-12, and IL-18 in repeated implantation failure after in vitro fertilization. *Fertil Steril* 2004; 81:59–65.
24 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.
25 Saito S: Cytokine network at the feto-maternal interface. *J Reprod Immunol* 2000; 47:87–103.
26 Tsuda H, Michimata T, Sakai M, Nagata K, Nakamura M, Saito S: A novel surface molecule of Th2- and Tc2-type cells, CRTH2 expression on human peripheral and decidual CD4+ and CD8+ T cells during the early stage of pregnancy. *Clin Exp Immunol* 2001; 123:105–111.
27 Michimata T, Tsuda H, Sakai M, Fujimura M, Nagata K, Nakamura M, Saito S: Accumulation of CRTH2-positive T-helper 2 and T-cytotoxic 2 cells at implantation sites of human decidua in a prostaglandin D(2)-mediated manner. *Mol Hum Reprod* 2002; 8:181–187.
28 Fallon PG, Jolin HE, Smith P, Emson CL, Townsend MJ, Fallon R, Smith P, McKenzie AN: IL-4 induces characteristic Th2 responses even in the combined absence of IL-5, IL-9, and IL-13. *Immunity* 2002; 17:7–17.
29 Lin Y, Ren L, Wang W, Di J, Zeng S, Saito S: Effect of TLR3 and TLR7 activation in uterine NK cells from non-obese diabetic (NOD) mice. *Reprod Immunol* 2009; 82:12–23.
30 Miyazaki S, Tsuda H, Sakai M, Hori S, Sasaki Y, Futatani T, Miyawaki T, Saito S: Predominance of Th2-promoting dendritic cells in early human pregnancy decidua. *J Leukoc Biol* 2003; 74:514–522.
31 Croy BA, Chantakru S, Esadeg S, Ashkar AA, Wei Q: Decidual natural killer cells: key regulators of placental development (a review). *J Reprod Immunol* 2002; 57:151–168.
32 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.
33 Santner-Nanan B, Peek MJ, Khanam R, Richarts L, Zhu E, Fazekas de St Groth B, Nanan R: Systemic increase in the ratio between Foxp3+ and IL-17-producing CD4+ T cells in healthy pregnancy but not in preeclampsia. *J Immunol* 2009; 183:7023–7030.
34 Ito M, Nakashima A, Hidaka T, Okabe M, Bac ND, Ina S, Yoneda S, Shiozaki A, Sumi S, Tsuneyama K, Nikaido T, Saito S: A role for IL-17 in induction of an inflammation at the fetomaternal interface in preterm labour. *J Reprod Immunol* 2010; 84:75–85.
35 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.
36 Pongcharoen S, Supalap K: Interleukin-17 increased progesterone secretion by JEG-3 human choriocarcinoma cells. *Am J Reprod Immunol* 2009; 61:261–264.
37 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.
38 Somerset DA, Zheng Y, Kilby MD, Sansom DM, Drayson MT: Normal human pregnancy is associated with an elevation in the immune suppressive CD25+ CD4+ regulatory T-cell subset. *Immunology* 2004; 112:38–43.
39 Zhao JX, Zeng YY, Liu Y: Fetal alloantigen is responsible for the expansion of the CD4(+)CD25(+) regulatory T cell pool during pregnancy. *J Reprod Immunol* 2007; 75:71–81.
40 Tilburgs T, Roelen DL, van der Mast BJ, de Groot-Swings GM, Kleijburg C, Scherjon SA, Claas FH: Evidence for a selective migration of fetus-specific CD4+CD25bright regulatory T cells from the peripheral blood to the decidua in human pregnancy. *J Immunol* 2008; 180:5737–5745.

© 2010 John Wiley & Sons A/S

41 Tilburgs T, Scherjon SA, van der Mast BJ, Haasnoot GW, Versteeg-V D Voort-Maarschalk M, Roelen DL, van Rood JJ, Claas FH: Fetal-maternal HLA-C mismatch is associated with decidual T cell activation and induction of functional T regulatory cells. *J Reprod Immunol* 2009; 82:148–157.
42 Nakashima A, Ito M, Shima T, Bac ND, Hidaka T, Saito S: Accumulation of IL-17-positive cells in decidua of inevitable abortion cases. *Am J Reprod Immunol*. in press.
43 Wang WJ, Hao CF, Yi Lin, Yin GJ, Bao SH, Qiu LH, Lin QD: Increased prevalence of T helper 17 (Th17) cells in peripheral blood and decidua in unexplained recurrent spontaneous abortion patients. *J Reprod Immunol* 2010; 84:164–170.
44 Arruvito L, Billordo A, Capucchio M, Prada ME, Fainboim L: IL-6 trans-signaling and the frequency of CD4+FOXP3+ cells in women with reproductive failure. *J Reprod Immunol* 2009; 82:158–165.
45 Yang H, Qiu L, Chen G, Ye Z, Lü C, Lin Q: Proportional change of CD4+CD25+ regulatory T cells in decidua and peripheral blood in unexplained recurrent spontaneous abortion patients. *Fertil Steril* 2008; 89:656–661.
46 Pasare C, Medzhitov R: Toll pathway-dependent blockade of CD4+CD25+ T cell-mediated suppression by dendritic cells. *Science* 2003; 299:1033–1036.
47 Yang Y, Huang CT, Huang X, Pardoll DM: Persistent Toll-like receptor signals are required for reversal of regulatory T cell-mediated CD8 tolerance. *Nat Immunol* 2004; 5:508–515.
48 Shima T, Sasaki Y, Itoh M, Nakashima A, Ishii N, Sugamura K, Saito S: Regulatory T cells are necessary for implantation and maintenance during early stage of pregnancy, but not necessary during late stage of pregnancy in allogeneic mice. *J Reprod Immunol* in press.
49 Zenclussen AC, Gerlof K, Zenclussen ML, Ritschel S, Zambon Bertoja A, Fest S, Hontsu S, Ueha S, Matsushima K, Leber J, Volk HD: Regulatory T cells induce a privileged tolerant microenvironment at the fetal-maternal interface. *Eur J Immunol* 2006; 36:82–94.
50 Makhseed M, Raghupathy R, El-Shazly S, Azizieh F, Al-Harmi JA, Al-Azemi MM: Pro-inflammatory maternal cytokine profile in preterm delivery. *Am J Reprod Immunol* 2003; 49:308–318.
51 Gargano JW, Holzman C, Senagore P, Thorsen P, Skogstrand K, Hougaard DM, Rahbar MH, Chung H: Mid-pregnancy circulating cytokine levels, histologic chorioamnionitis and spontaneous preterm birth. *J Reprod Immunol* 2008; 79:100–110.
52 Germain SJ, Sacks GP, Sooranna SR, Sargent IL, Redman CW: Systemic inflammatory priming in normal pregnancy and preeclampsia: the role of circulating syncytiotrophoblast microparticles. *J Immunol* 2007; 178:5949–5956.
53 Sasaki Y, Darmochwal-Kolarz D, Suzuki D, Sakai M, Ito M, Shima T, Shiozaki A, Rolinski J, Saito S: Proportion of peripheral blood and decidual CD4(+) CD25(bright) regulatory T cells in pre-eclampsia. *Clin Exp Immunol* 2007; 149:139–145.
54 Steinborn A, Haensch GM, Mahnke K, Schmitt E, Toermer A, Meuer S, Sohn C: Distinct subsets of regulatory T cells during pregnancy: is the imbalance of these subsets involved in the pathogenesis of preeclampsia? *Clin Immunol* 2008; 129:401–412.
55 Hu D, Chen Y, Zhang W, Wang H, Wang Z, Dong M: Alteration of peripheral CD4+CD25+ regulatory T lymphocytes in pregnancy and pre-eclampsia. *Acta Obstet Gynecol Scand* 2008; 87:190–194.
56 Paeschke S, Chen F, Horn N, Fotopoulou C, Zambon-Bertoja A, Sollwedel A, Zenclussen ML, Casalis PA, Dudenhausen JW, Volk HD, Zenclussen AC: Pre-eclampsia is not associated with changes in the levels of regulatory T cells in peripheral blood. *Am J Reprod Immunol* 2005; 54:384–389.
57 Acosta-Rodriguez EV, Napolitani G, Lanzavecchia A, Sallusto F: Interleukins 1beta and 6 but not transforming growth factor-beta are essential for the differentiation of interleukin 17-producing human T helper cells. *Nat Immunol* 2007; 8:942–949.
58 Redman CW, Sacks GP, Sargent IL: Preeclampsia: an excessive maternal inflammatory response to pregnancy. *Am J Obstet Gynecol* 1999; 180:499–506.
59 Levine RJ, Lam C, Qian C, Yu KF, Maynard SE, Sachs BP, Sibai BM, Epstein FH, Romero R, Thadhani R, Karumanchi SA: Soluble endoglin and other circulating antiangiogenic factors in preeclampsia. *N Engl J Med* 2006; 355:992–1005.

© 2010 John Wiley & Sons A/S