Decidual and peripheral blood CD4+CD25+ regulatory T cells in early pregnancy subjects and spontaneous abortion cases

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Human pregnancy represents a situation of semiallograft to maternal host. Therefore, it has been reported that tolerance to the fetal allograft represents a mechanism for maintaining a pregnancy. CD4+CD25bright regulatory T cells are known to play an important role in the development and maintenance of tolerance in peripheral tissues. However, the potential role of CD4+CD25bright T cells in maintaining human pregnancy has not been reported. In this study, we show that early human pregnancy decidua contains an abundance of CD4+CD25bright T cells, which express CD152(CTLA-4) at a high level. CD4+CD25bright T cells mediate potent inhibition of autologous T-cell proliferation by anti-CD3 stimulation. Furthermore, these cells inhibit the proliferation of autologous CD4+CD25− T cells in a dose-dependent fashion. This suppressive function of decidual CD4+CD25+ T cells required cell-to-cell contact. The proportion of decidual CD4+CD25bright T cells was significantly lower in specimens from spontaneous abortion compared to those from specimens from induced abortions. These results suggest that decidual CD4+CD25bright T cells contribute to the mechanisms mediating maternal immune tolerance of conceptus antigens and therefore might contribute to the maintenance of pregnancy.

Key words: abortion/decidua/pregnancy/regulatory T cells/tolerance

Introduction

Sir Peter Medawar introduced the concept that human pregnancy represents a condition of semiallograft to maternal host because of the presence of paternally inherited gene products and the production of unique differentiation antigens (Medawar, 1953). Therefore, the process of implantation may include mechanisms preventing allograft rejection (Medawar, 1953; Thellin *et al*., 2000).

T-cell-mediated immunoregulation is one of the main mechanisms responsible for maintaining antigen-specific operational tolerance *in vivo* (Graca *et al*., 2002; Karim *et al*., 2002; Bach, 2003; Bluestone and Abbans, 2003; Word and Sakaguchi, 2003). Accumulating evidence from both experimental and clinical studies indicates that the balance between regulation and deletion of responder T cells controls immune responsiveness after organ or cell transplantation (Graca *et al*., 2002; Karim *et al*., 2002; Bach, 2003; Bluestone and Abbans, 2003; Word and Sakaguchi, 2003). Recently it has been demonstrated that regulatory T (Treg) cells have a role in the induction of transplantation tolerance (Graca *et al*., 2002; Karim *et al*., 2002; Bach, 2003; Bluestone and Abbans, 2003; Word and Sakaguchi, 2003). Of these Treg cells, CD4+CD25+ Treg cells play a role in the induction of tolerance (Sakaguchi *et al*., 1995; Taylor *et al*., 2001; Beacher-Allan *et al*., 2001; Jonuleit *et al*., 2001; Levings *et al*., 2001; Ng *et al*., 2001; Stephens *et al*., 2001; Cohen *et al*., 2002; Hoffmann *et al*., 2002; Word and Sakaguchi, 2003). CD4+CD25+ Treg are shown to have potent regulatory properties in both the induction and maintenance phases of *in vivo* tolerance to alloantigens in mice (Sakaguchi *et al*., 1995; Taylor *et al*., 2001; Cohen *et al*., 2002; Hoffmann *et al*., 2002) and humans (Beacher-Allan *et al*., 2001;

Jonuleit *et al*., 2001; Levings *et al*., 2001; Ng *et al*., 2001; Stephens *et al*., 2001). Mouse and human CD4+ T cells that have the most potent regulatory properties express high and sustained levels of CD25, in contrast to recently activated T cells, which express CD25 only transiently and at a lower level. Cytotoxic T-lymphocyte antigen 4 (CTLA4;CD152) is expressed constitutively by CD4+CD25+ Treg cells, and CTLA-4 blockade prevents regulation by CD4+CD25+ Treg cells in *in vitro* and *in vivo* transplantation models (Read *et al*., 2000; Takahashi *et al*., 2000).

In the present study, we compared the proportion of decidual and peripheral blood CD4+CD25bright Treg in the setting of normal early pregnancy and spontaneous abortion. In addition, we performed functional analysis on decidual CD4+CD25+ Treg, to evaluate their antiproliferative effects on activated CD4+CD25− autologous T cells.

Materials and methods

Blood and tissue samples

Heparinized venous blood was obtained from non-pregnant healthy women ($n = 10$), and women in early pregnancy ($n = 19$). Similarly, heparinized venous blood was also obtained from patients with early abortion ($n = 9$). Decidual samples were obtained from patients with induced abortion, representing early pregnant deciduas, and from patients with spontaneous abortion, representing abortion samples. All samples were from women in the fifth to ninth gestational week. Decidual mononuclear cells (leukocytes) were purified by the Ficoll-Hypaque method after homogenization and filtration through a 32 µm nylon mesh as previously reported (Saito *et al*., 1992; Tsuda *et al*., 2002). Decidual tissues were not enzymatically digested, to prevent the possibility that enzymatic treatment might affect the fluorescence intensity of surface antigens.

Peripheral blood mononuclear cells were isolated by the standard Ficoll-Hypaque method. Informed consent was obtained from all patients. All of the sampling and use of the tissues for this study were approved by Toyama Medical and Pharmaceutical University Ethics Committee.

Flow cytometry

In this study, we used mouse monoclonal antibodies (mAb) including anti-CD4 mAb (fluorescein isothiocyanate; Becton Dickinson, USA), anti-CD25 mAb (PE, Becton Dickinson) and anti-CD152 (CTLA-4) mAb (Biotin-PC5; Becton Dickinson). To stain cells for intracellular markers, isolated mononuclear cells were first stained with anti-CD4 (2 µg/ml) and CD25 mAb (2 µg/ml), then permeabilized by incubating for 10 min with permeabilizing solution buffer (Becton Dickinson, dilute 10× solution 1:10 in deionized water) and then stained with anti-CD152 mAb (2 µg/ml). Flow cytometry analysis was performed on a FACS Callibur using CellQuest software (Becton Dickinson). For the analysis, a real-time gate was set around the viable lymphocytes based on their forward scatter/side scatter profile, avoiding monocyte and granulocyte populations. The proportions of CD4+CD25bright, CD4+CD25dim, CD4−CD25bright and CD4−CD25dim cells in lymphocytes were calculated. The proportions for intracellular CD152+ and extracellular CD152+ cells in CD4+CD25bright and CD4+CD25dim cells were also calculated.

Purification of CD4+ T-cell subsets

Isolated mononuclear cells were purified using magnetic goat anti-mouse IgG beads and MACS-negative and -positive selection columns (Miltenyi Biotic, UK). Isolated mononuclear cells were incubated with mouse monoclonal antibodies, anti-CD8 (2 µg/ml), anti-CD14 (2 µg/ml), anti-CD56 (2 µg/ml) and anti-CD19 (2 µg/ml) (all from Becton Dickinson), and then incubated with magnetic goat anti-mouse IgG beads. CD4+ T cells were purified using MACS columns for negative selection. The purity of CD4+ T cells purified by this method was up to 98%, confirmed by flow cytometric analysis. Purified CD4+ T cells were then incubated with anti-CD25 mouse monoclonal antibodies and CD4+CD25+ T cells were separated from CD4+CD25− T cells using MACS columns. The purity of CD4+CD25+ T cells was >95%.

Proliferation assays

Flat-bottom 96-well plates (Becton Dickinson) were pre-coated with 1 µg/ml of anti-CD3 mAb (NU-T3; Nichirei, Japan) for 4 h at 37°C. Purified T-cell subsets were cultured in triplicate wells at a density of 5×10^4 T cells/well in 200 µl of culture medium [Roswell Park Memorial Institute Medium 1640 (Sigma–Aldrich, Japan), supplemented with penicillin/streptomycin, and 10% heat-inactivated fetal calf serum]. For co-stimulation assays, irradiated (20 Gy) autologous peripheral blood mononuclear cells were added as antigen-presenting cells (APC). Cells were cultured for 96 h at 37°C in 5% CO_2, and during the last 18 h [^{3}H]thymidine (0.5 µCi/well, Amersham Pharmacia Biotech, Japan) and [^{3}H]thymidine incorporation was measured. Proliferative responses are expressed as the mean ± SD [^{3}H]thymidine incorporation (cpm) of triplicate samples.

Direct suppression assays

For direct suppression assays, 5×10^4 CD4+CD25− T cells were stimulated with anti-CD3 mAb and APC, either in the absence or presence of increasing numbers (0.5×10^4, 2.5×10^4, 5×10^4) of CD4+CD25+ T cells, resulting in responder:suppressor ratios of 1:0, 1:0.1, 1:0.5 and 1:1. Cells were cultured in the same way as described for the proliferation assay. The direct suppressive effect of CD4+CD25+ T cells was evaluated by measuring [^{3}H]thymidine incorporation.

Transwell experiments

CD4+CD25− T cells were co-cultured with CD4+CD25+ T cells using a semi-permeable transwell membrane (Nunc, Denmark). Both the lower and upper chambers of the transwell plate were coated with anti-CD3 mAb for 4 h. To the lower chamber, 5×10^4 CD4+CD25− T cells and 5×10^4 irradiated APC were added. To the upper chamber, 5×10^4 irradiated APC were added in the presence of medium alone, or together with 5×10^4 CD4+CD25− T cells or 5×10^4 CD4+CD25+ T cells. At the same time, 5×10^4 CD4+CD25− T cells and 5×10^4 irradiated APC were cultured with 5×10^4 CD4+CD25− T cells or 5×10^4

CD4+CD25+ T cells without a semi-permeable transwell membrane. The proliferative responses were assessed as described for the proliferation assays.

Statistical analysis

Data were analysed using the Mann–Whitney *U*-test and one-way ANOVA. $P < 0.05$ was considered significant.

Results

CD25 expression in peripheral and decidual lymphocytes

The subsets of lymphocytes in the decidua were different from those in peripheral blood, as previously reported (Nishikawa *et al.*, 1991). The CD4+ T cell population was markedly lower in the decidua because CD16−CD56bright NK cells were the dominant subset (60–70% of lymphocytes in the decidua during early pregnancy).

CD25 expression was observed in a subset of CD4+ T cells (Figure 1). We subdivided CD25+CD4+ T cells into bright and dim populations (CD25bright and CD25dim; Figure 1). This is in keeping with previous reports that regulatory T cells preferentially reside within the CD4+CD25bright T cell population, and activated T cells express lower levels of CD25 than that expressed by regulatory T cells (Kuniyasu *et al.*, 2000; Beacher-Allan *et al.*, 2001; Cao *et al.*, 2003).

The percentages of CD25-expressing cells in the CD4+ cell population were compared between decidual lymphocytes and peripheral blood lymphocytes. The percentages of CD4+ cells and CD25dim cells in the CD4+ cell population of peripheral blood lymphocytes did not differ significantly between non-pregnant individuals, normal early pregnant women and patients with spontaneous abortion (Table I). However, the percentage of peripheral blood CD4+CD25dim cells (activated CD4+ T cells) in lymphocytes was significantly lower in the setting of early pregnancy than in non-pregnant women and women with spontaneous abortion ($P < 0.01$ and $P < 0.05$ respectively; Table II).

The percentage of CD25bright cells in peripheral CD4+ cells in women with early pregnancy was significantly higher than in non-pregnant women, while the percentage of CD25bright cells in peripheral CD4+ T cells from patients with spontaneous abortion was the same as non-pregnant women (Table I).

In the setting of normal pregnancy, the percentage of CD25bright cells in the CD4+ T cell population was significantly higher in the decidua than in the peripheral blood (Figure 1 and Table I). However, a higher frequency of CD25bright cells in the CD4+ T cell population was not seen in patients with spontaneous abortion (Table I). The percentage of CD4−CD25 dim cells in the peripheral blood lymphocytes, including CD8+ T cells, CD16−CD56bright NK cells and CD16+CD56dim NK cells was significantly higher in women with spontaneous abortion than in non-pregnant women or women during early pregnancy ($P = 0.013$, $P = 0.0004$ respectively) (Table II). Similarly, the percentage of CD4−CD25dim cells in the decidual lymphocytes in women with spontaneous abortion tended to be higher than in women during early pregnancy, but the difference was not significant ($P = 0.06$).

Surface and intracellular expression of CD152 (CTLA-4) on CD4+CD25bright T cells

Recent studies have shown that CD152 is up-regulated on mouse and human regulatory T cells (Chambers *et al.*, 2001). CD152 is constitutively expressed in the cytoplasm of regulatory T cells, but after activation by certain antigens, CD152 is expressed on their surface. Therefore, we analysed the surface and intracellular expression of CD152 on CD4+CD25bright T cells in peripheral blood and decidua.

Peripheral blood   Decidua
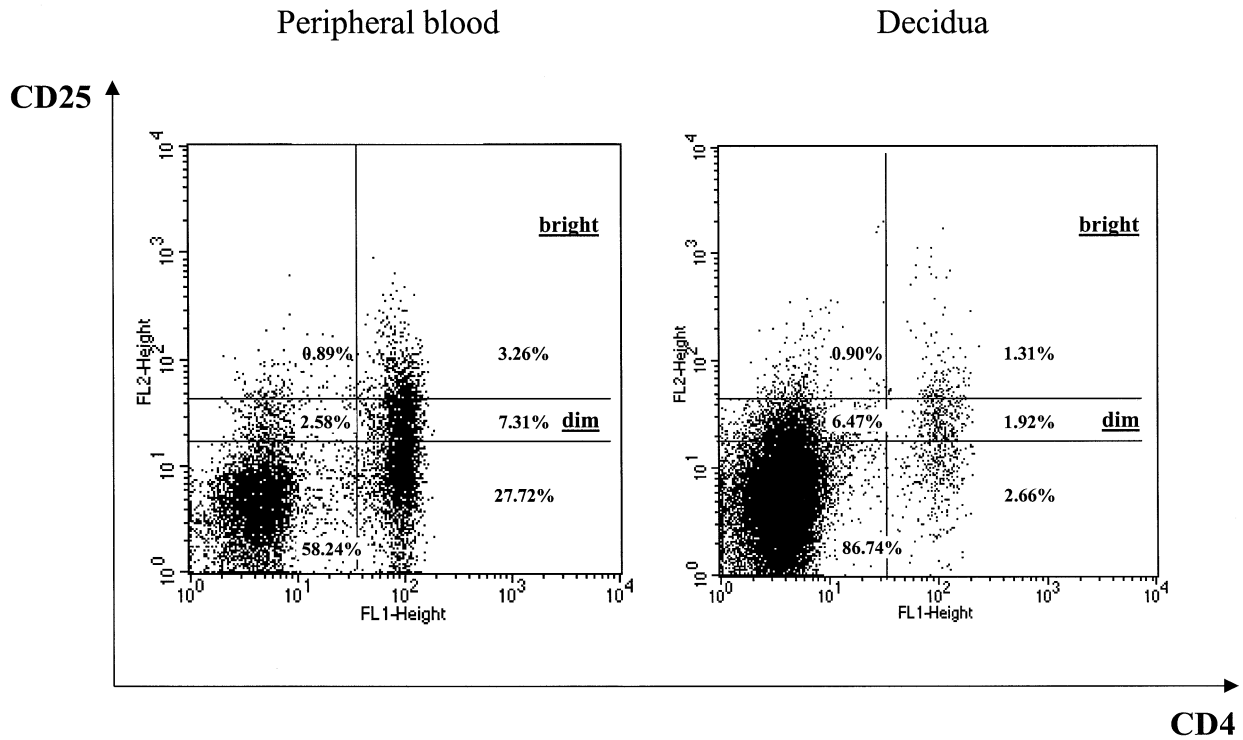
Figure 1. CD4$^+$CD25$^+$ T cells can be classified into CD4$^+$CD25^{bright} T cells and CD4$^+$CD25^{dim} T cells. Representative FACS stainings of peripheral blood mononuclear cells and decidual leukocytes from one of the pregnant women are shown. Markers were set according to the isotype IgG, and lymphocytes were classified into CD4$^+$ cells (right square), CD4$^-$ cells (left square), CD25^{bright} cells (upper square), CD25^{dim} cells (middle square) and CD25$^-$ (lower square). The percentage of CD4$^+$CD25^{bright} T cells is indicated in the upper right quadrant, and the percentage of CD4$^+$CD25^{dim} T cells is indicated in the middle right quadrant. Upper left quadrant shows CD4$^-$CD25^{bright} cells and middle left quadrant shows CD4$^-$CD25^{dim} cells.

Table I. Population of CD4$^+$ cells in lymphocytes and populations of CD25^{bright} cells and CD25^{dim} cells in CD4$^+$ cells

	CD4$^+$/lymphocyte (%)	CD4$^+$CD25^{bright}/CD4$^+$ (%)	CD4$^+$CD25^{dim}/CD4$^+$ (%)
Non-pregnant (n = 10)			
PBL	44.77 ± 7.97	6.50 ± 0.59	23.08 ± 4.97
Early pregnancy (n = 19)			
PBL	38.29 ± 6.29	8.51 ± 2.48*†	19.08 ± 6.32
Decidua	5.98 ± 3.01	21.84 ± 2.92**††	23.72 ± 4.29
Spontaneous abortion (n = 9)			
PBL	42.28 ± 10.34	5.66 ± 1.58	23.86 ± 5.08
Decidua	4.87 ± 2.29	7.14 ± 1.85	22.70 ± 14.46

*$P < 0.05$ versus non-pregnant PBL; †$P < 0.001$ versus spontaneous abortion PBL; **$P < 0.0001$ versus non-pregnant PBL; ††$P < 0.0001$ versus spontaneous abortion decidua.
PBL = peripheral blood.

Table II. The population of CD4$^+$CD25^{bright} cells, CD4$^+$CD25^{dim} cells, CD4$^-$CD25^{bright} cells and CD4$^-$CD25^{dim} cells in peripheral blood lymphocytes and decidual lymphocytes

	CD4$^+$CD25^{bright}/ lymphocytes	CD4$^+$CD25^{dim}/ lymphocytes	CD4$^-$CD25^{bright}/ lymphocytes	CD4$^-$CD25^{dim}/ lymphocytes	CD25$^+$/ lymphocytes
Non-pregnant (n = 10)					
Peripheral blood	2.91 ± 0.27	10.33 ± 2.22	0.49 ± 0.06	3.47 ± 0.71	16.37 ± 1.96
Early pregnancy (n = 19)					
Peripheral blood	3.26 ± 0.95*	7.31 ± 2.42***	0.89 ± 0.59	2.58 ± 1.66	14.03 ± 3.75
Decidua	1.31 ± 0.17**	1.96 ± 0.90	0.90 ± 0.82	6.47 ± 3.64	10.04 ± 4.20
Spontaneous abortion (n = 9)					
Peripheral blood	2.39 ± 0.65	10.09 ± 2.15	0.60 ± 0.20	7.20 ± 2.41†	18.83 ± 3.01††
Decidua	0.35 ± 0.09	1.30 ± 0.72	0.16 ± 0.09	12.60 ± 6.26	14.59 ± 6.09

*$P < 0.05$ vs. spontaneous abortion (sp-ab) PBL; **$P < 0.0001$ vs. sp-ab decidua; ***$P < 0.01$ vs. non-pregnant PBL and early pregnancy PBL;
†$P < 0.01$ vs. non-pregnant PBL or early pregnant PBL; ††$P < 0.01$ vs. early pregnancy PBL.

Both peripheral blood and decidual CD4+CD25bright T cells expressed intracellular CD152 at >90% (Table III). In early pregnancy decidua, the percentage of surface CD152+ in CD4+CD25bright T cells was significantly higher than in peripheral blood CD4+CD25bright T cells (Table III). In the setting of spontaneous abortion, there was no significant difference in the surface CD152 expression between decidual CD4+CD25bright T cells and peripheral blood CD4+CD25bright T cells (Table III). However, both peripheral blood and decidual CD4+CD25dim T cells expressed intracellular and surface CD152 at a low frequency (Table III).

Table III. Surface and intracellular CD152 expression on CD4+CD25bright T cells in early pregnancy and spontaneous abortion

	CD4+CD25+CD152+/CD4+CD25+ (%)	
	Intracellular	Surface
Non-pregnant (*n* = 10)		
Peripheral blood		
CD4+CD25bright	92.81 ± 1.72	1.01 ± 0.73
CD4+CD25dim	6.78 ± 1.93	0.73 ± 0.49
Early pregnancy (*n* = 19)		
Peripheral blood		
CD4+CD25bright	96.0 ± 1.01	0.99 ± 0.77
CD4+CD25dim	6.83 ± 1.14	0.76 ± 0.32
Decidua		
CD4+CD25bright	97.78 ± 3.22	5.20 ± 3.80*
CD4+CD25dim	5.74 ± 0.75	1.16 ± 0.74
Spontaneous abortion (*n* = 9)		
Peripheral blood		
CD4+CD25bright	96.0 ± 1.21	1.11 ± 0.80
CD4+CD25dim	5.20 ± 1.37	0.81 ± 0.57
Decidua		
CD4+CD25bright	91.71 ± 3.82	3.34 ± 3.18
CD4+CD25dim	5.83 ± 1.46	1.09 ± 0.95

*$P < 0.05$ vs. early pregnant PBL.

### CD4+CD25+ regulatory T cells become anergic by anti-CD3 stimulation

Figure 2 shows the proliferative responses of the different isolated CD4+ T-cell subsets with anti-CD3 stimulation and irradiated APC addition. CD4+CD25+ T cells were less responsive than either CD4+CD25− T cells or total CD4+ T cells ($P = 0.0003$ for CD4+CD25− T cells, $P = 0.0008$ for total CD4+ T cells in the decidua, $P = 0.0002$ for CD4+CD25− T cells, $P = 0.03$ for total CD4+ T cells in peripheral blood). After removing CD4+CD25+ T cells from the total CD4+ T cell population, the proliferative response of the remaining CD4+CD25− T cells was significantly greater ($P = 0.0095$ for total CD4+ T cells in the decidua, $P = 0.02$ for total CD4 T cells in peripheral blood). These findings suggest that with the anti-CD3 stimulation, CD4+CD25+ T cells become not only anergic, but also actively suppress the proliferative response of other CD4+ T cells.

### CD4+CD25+ regulatory T cells have directly and dose-dependently suppressive potential

To investigate the suppressive potential of CD4+CD25+ T cells, co-culture experiments were performed. The response of CD4+CD25− T cells to anti-CD3 antibodies and APC was significantly suppressed to 50% ($P < 0.0001$ for the deciduas, $P = 0.0059$ for peripheral blood) by the addition of CD4+CD25+ T cells at a 1:1 ratio of the two cell populations (Figure 3). The suppressive effect of CD4+CD25+ T cells was dose dependent and the use of CD4+CD25+ T cells at a responder:CD4+CD25+ cell ratio of 1:0.1 did not lead to suppression (Figure 3).

### The suppressive effect of CD4+CD25+ T cells requires direct cell–cell contact

When cell–cell contact between peripheral blood CD4+CD25− T cells and decidual CD4+CD25+ T cells (1:1) was prevented by a semi-
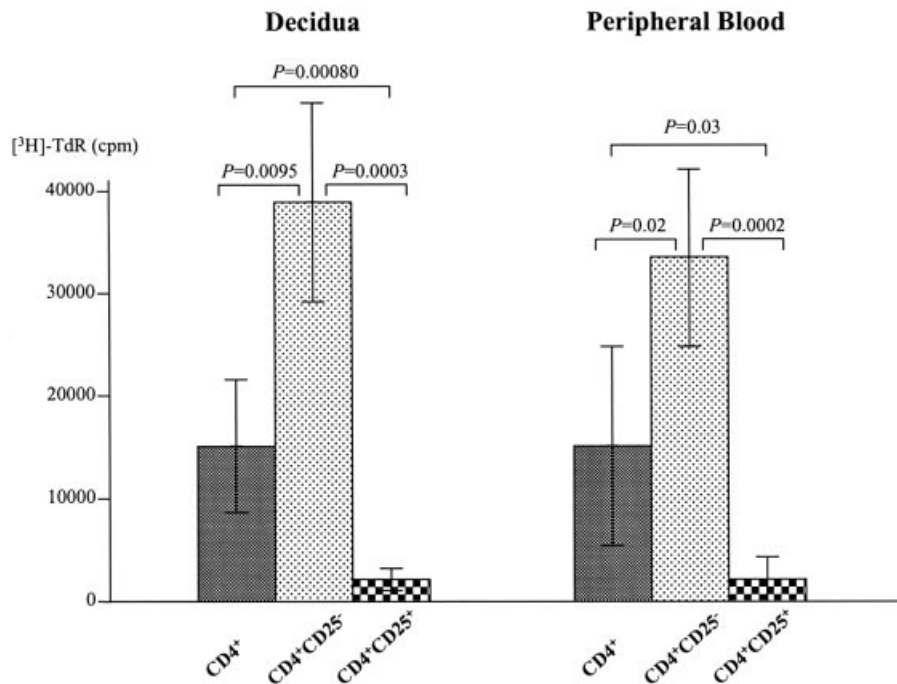
Figure 2. Decidual and peripheral blood CD4+CD25+ cells are hyporesponsive to T-cell stimulation. CD4+ cells (—), CD4+CD25− cells (—) or CD4+CD25+ cells (—) were stimulated in the presence of irradiated autologous APC with anti-CD3 antibody for 4 days. Proliferation was measured by [³H]thymidine incorporation. The T-cell responses were increased by removing CD4+CD25+ T cells from the total CD4+ T cell population (—: CD4+CD25−). The proliferative responses of CD4+CD25+ T cells to anti-CD3 were considerably lower than those of CD4+ T cells and CD4+CD25− T cells.

Decidua   ## Peripheral Blood
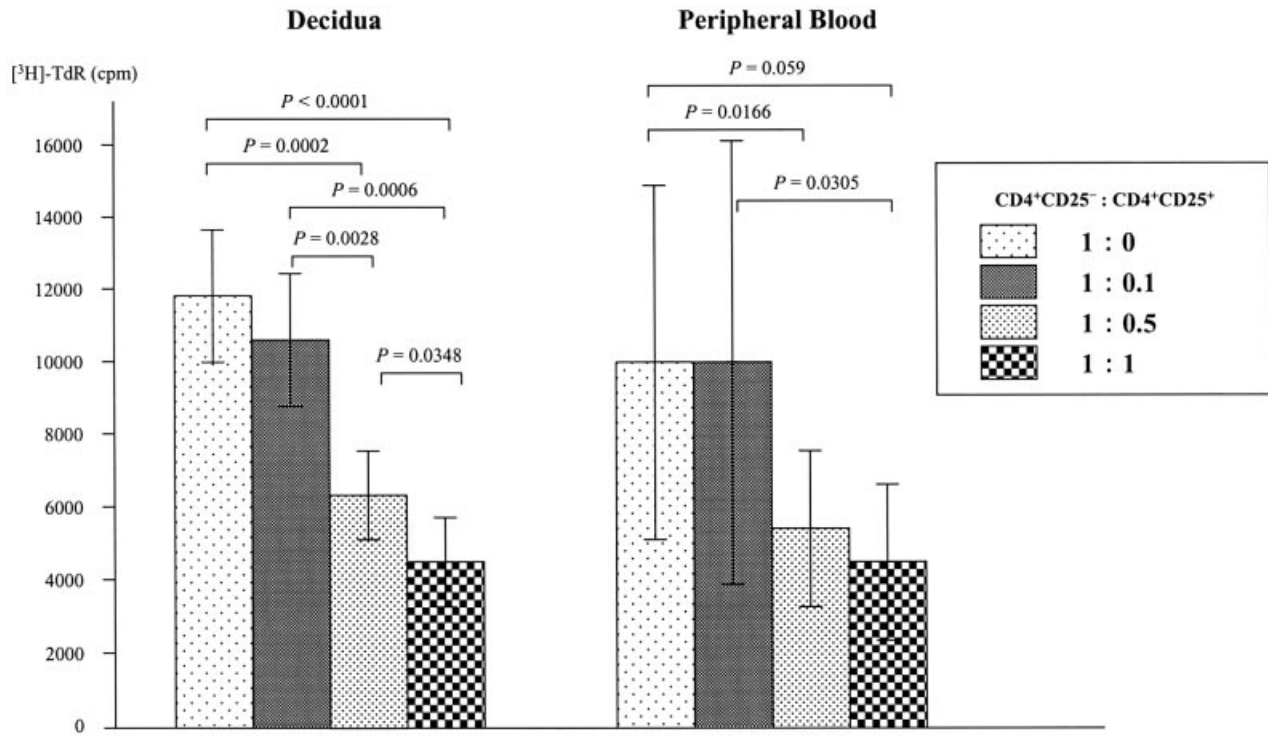
Figure 3. The suppressive effect of decidual and peripheral blood CD4+CD25+ cells is cell ratio dependent. Increasing numbers of CD4+CD25+ T cells were seeded into a fixed number of autologous CD4+CD25− responder T cells. A dose-dependent suppression of DNA synthesis by decidual and peripheral blood CD4+CD25+ T cells was observed.

Transwell culture (Decidua)
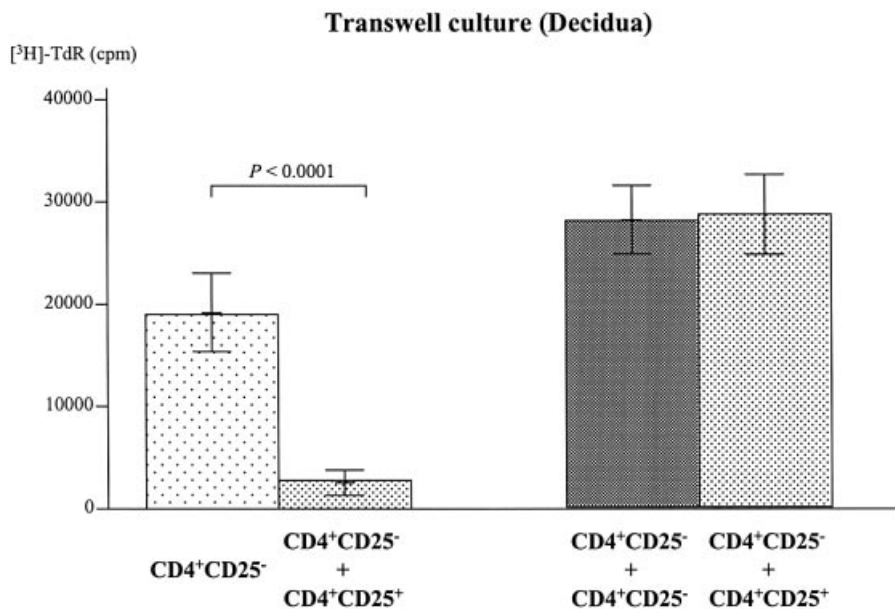
Figure 4. Cell contact is necessary for suppression. CD4+CD25− T cells were stimulated with anti-CD3 and APC in the presence or absence of CD4+CD25+ T cells, added directly in co-culture or in the upper chamber of a transwell plate together with anti-CD3 and APC. When cell–cell contact between CD4+CD25+ T cells was prevented by a semi-permeable transwell membrane (right panel), the suppressive effects of CD4+CD25+ T cells were abrogated.

permeable transwell membrane, the suppressive effects of CD4+CD25+ T cells were abrogated (Figure 4). Similar results were observed in the peripheral blood CD4+CD25+ T cells (data not shown). These results suggest that cell contact is necessary for the inhibitory effects of CD4+CD25+.

Discussion

This is the first study to demonstrate the presence and abundance of CD4+CD25bright Treg in the decidua during early pregnancy. We first reported that the expression of CD25 on decidual CD4+ T cells is

significantly higher compared to that for peripheral blood CD4^{+} T cells, but the expression rate for CD4^{+}CD25^{+} T cells in CD4^{+} T cells is rather low compared to those of CD4^{+}CD69^{+} and CD4^{+}HLA-DR^{+} T cells (Saito *et al.*, 1992). In contrast, Chao *et al.* (2002) reported that the expression of CD25 is decreased on decidual CD4^{+} and CD8^{+} T cells in normal pregnancy. However, the mean fluorescence intensity of CD25 on decidual CD4^{+} T cells was almost twice that on peripheral blood CD4^{+} T cells (Chao *et al.*, 2002), suggesting that CD4^{+}CD25^{bright} T cells are increased in the decidua, even though the total populations of CD4^{+}CD25^{dim} T cells and CD4^{+}CD25^{bright} T cells are decreased. It has been reported that the regulatory CD4^{+} T cells in humans preferentially reside within the CD4^{+}CD25^{bright} T-cell population, while the CD4^{+}CD25^{int} T-cell population holds a majority of normal activated T cells (Kuniyasu *et al.*, 2000; Beacher-Allan *et al.*, 2001; Chao *et al.*, 2002). These reports suggest that CD4^{+}CD25^{bright} T cells are increased in the early human pregnancy decidua.

In this study, the gate for CD25 was deliberately set high to allow for isolation of regulatory T cells. The majority of CD4^{+}CD25^{bright} Treg cells are activated memory T cells expressing CD45RO, CD69, HLA-DR, CD122, CD58, CD71 and CTLA-4 (CD152) (Karim *et al.*, 2002; Bach, 2003; Bluestone and Abbans, 2003; Word and Sakaguchi, 2003). In this study, decidual CD4^{+}CD25^{bright} T cells expressed CD152 (CTLA-4). Expression of these antigens on decidual CD4^{+}CD25^{bright} T cells are a quite similar to that of CD4^{+}CD25^{+} Treg, suggesting that decidual CD4^{+}CD25^{bright} T cells have immunoregulatory functions.

CTLA-4 is known to be an important negative regulator of T-cell function (Chambers *et al.*, 2001). CD4^{+}CD25^{+} Treg cells have been shown to express CTLA-4. Newly translated CTLA-4 emerge from the Golgi apparatus in secretory vesicles, trafficking to the endosomal compartment or directly to the cell surface. Upon T-cell receptor (TCR) stimulation, CTLA-4 is phosphorylated by protein kinases associated with the TCR, resulting in surface stabilization of CTLA-4. Binding of both B7-1 (CD80) and B7-2 (CD86) by CTLA-4 has considerably higher affinity than that of CD28 binding. Surface CTLA-4 can compete with CD28 for B7 binding, resulting in TCR engagement in the absence of B7 expression on antigen-presenting cells (Chambers *et al.*, 2001). This stimulation does not result in T-cell proliferation or induction of T-cell anergy (Chambers *et al.*, 2001). CTLA-4 also delivers inhibitory signals which prevent T-cell activation. In the decidua during early pregnancy, expression of surface CTLA-4 on CD4^{+}CD25^{bright} T cells was increased, indicating that decidual CD4^{+}CD25^{bright} T cells are already stimulated upon TCR, perhaps by fetal antigens.

Next, we analysed whether functional regulatory CD4^{+}CD25^{+} T cells exist in the decidua during early human pregnancy. The proliferative response of decidual CD4^{+}CD25^{+} T cells to anti-CD3 stimulation in the presence of autologous antigen-presenting cells was considerably lower than those of CD4^{+}CD25^{−} T cells. When decidual CD4^{+}CD25^{+} cells were co-cultured with peripheral blood CD4^{+}CD25^{−} cells, the responses to anti-CD3 antibody were significantly suppressed. To determine whether the suppressive function of decidual CD4^{+}CD25^{+} cells is mediated by the secretion of soluble factors, a transwell system was used. Based on the findings, the suppressive function of decidual CD4^{+}CD25^{+} T cells requires cell–cell contact. These characteristics of decidual CD4^{+}CD25^{+} T cells are quite similar to those of peripheral blood CD4^{+}CD25^{+} Treg cells. Recently, a unique population of extrathymically derived αβTCR^{+}CD4^{−}CD8^{−} B220^{low}CD25^{+} T cells with regulatory functions has been described in the mouse female genital tract (Johansson and Lycke, 2003). However, we could not detect these phenotypic cells in the human decidua.

There are several mechanisms that might explain the enrichment of CD4^{+}CD25^{+} Treg in the decidua. Chemokines have recently been described that attract CD4^{+}CD25^{+} T cells carrying the chemokine receptors CCR4 and CCR8. Interestingly, the number of CCR4-expressing T cells is increased in the decidua during early human pregnancy, and the chemotactic factor for CCR4, TARC (CCL17), is expressed on trophoblasts, endometrial gland cells, and uterine epithelial cells (Tsuda *et al.*, 2002). CD4^{+}CD25^{+} Treg cells might be recruited to the materno-fetal interface, at least in part, through a TARC-mediated pathway. Ho *et al.* (1996) reported that the population of CD3^{+}CD25^{+} T cells decreases significantly in the endometrium of ectopic pregnancies compared to the endometrium of normal pregnancies. Therefore, a fetus or placenta might be necessary for the accumulation of CD3^{+}CD25^{+} T cells.

The proportion of decidual CD4^{+}CD25^{bright} NK cells expressing surface CTLA-4 in spontaneous abortion cases was decreased in this study. But Vassiliadou *et al.* (1999) reported that a significantly increased number of CD25^{+} T cells was detected in the decidua of spontaneous abortions based on immunohistochemical methods. These differences could be explained by the fact that activated CD4^{+}CD25^{dim} T cells were increased in the spontaneous abortion decidua. Indeed, Quack *et al.* (2001) reported that significantly more activated leukocytes were detected in the decidua of women with unexplained recurrent spontaneous abortion (RSA) who had a normal male karyotype compared to either a control group or a group of patients with RSA and abnormal chromosomes. However, the expression of CD4^{+}CD25^{dim} T cells and CD4^{+}CD25^{bright} cells was not determined in Vissiliadou's study. In the present study, the proportion of decidual CD4^{−}CD25^{dim} cells in the spontaneous abortion cases was increased (Table I; $P = 0.06$), but CD4^{+}CD25^{dim} cells in the decidua of spontaneous abortions were the same as in the decidua of induced abortions. These results suggested that activated decidual CD8^{+} T cells or NK cells might be increased in spontaneous abortion. Furthermore, the proportions of peripheral blood CD4^{+}CD25^{dim} and CD4^{−}CD25^{dim} cells in the spontaneous abortion cases were significantly increased compared to those in induced abortion subjects. These data suggest that activated CD4^{+} T cells, CD8^{+} T cells or NK cells might be increased in peripheral blood of spontaneous abortion cases. It can be speculated that decreased CD4^{+}CD25^{bright} regulatory T cells in abortion cases might induce maternal lymphocyte activation to the fetal allograft. Further studies are needed to identify the proportion of CD4^{+}CD25^{bright} T cells, CD4^{+}CD25^{dim} T cells, CD8^{+}CD25^{+} T cells and CD56^{+}CD25^{+} NK cells in those cases.

Our data showed that the proportion of CD4^{+}CD25^{bright} Treg cells is increased in therapeutic abortion decidua and is decreased in spontaneous abortion decidua. These CD4^{+}CD25^{bright} Treg cells might play a role in the maintenance of pregnancy.

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