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Evidence for a Selective Migration of Fetus-Specific CD4^{+}CD25^{bright} Regulatory T Cells from the Peripheral Blood to the Decidua in Human Pregnancy

Tamara Tilburgs,[1]*† Dave L. Roelen,* Barbara J. van der Mast,† Godelieve M. de Groot-Swings,† Carin Kleijburg,† Sicco A. Scherjon,† and Frans H. Claas*

During pregnancy, the maternal immune system has to tolerate the persistence of fetal alloantigens. Many mechanisms contribute to the prevention of a destructive immune response mediated by maternal alloreactive lymphocytes directed against the allogeneic fetus. Murine studies suggest that CD4^{+}CD25^{+} T cells provide mechanisms of specific immune tolerance to fetal alloantigens during pregnancy. Previous studies by our group demonstrate that a significantly higher percentage of activated T cells and CD4^{+}CD25^{bright} T cells are present in decidual tissue in comparison with maternal peripheral blood in human pregnancy. In this study, we examined the phenotypic and functional properties of CD4^{+}CD25^{bright} T cells derived from maternal peripheral blood and decidual tissue. Depletion of CD4^{+}CD25^{bright} T cells from maternal peripheral blood demonstrates regulation to third party umbilical cord blood cells comparable to nonpregnant controls, whereas the suppressive capacity to umbilical cord blood cells of her own child is absent. Furthermore, maternal peripheral blood shows a reduced percentage of CD4^{+}CD25^{bright}FOXP3^{+} and CD4^{+}CD25^{bright}HLA-DR^{+} cells compared with peripheral blood of nonpregnant controls. In contrast, decidual lymphocyte isolates contain high percentages of CD4^{+}CD25^{bright} T cells with a regulatory phenotype that is able to down-regulate fetus-specific and fetus-nonspecific immune responses. These data suggest a preferential recruitment of fetus-specific regulatory T cells from maternal peripheral blood to the fetal-maternal interface, where they may contribute to the local regulation of fetus-specific responses. ***The Journal of Immunology,* 2008, 180: 5737–5745.**

Many mechanisms are suggested to be involved in maternal immune tolerance and immunologic acceptance of the allogeneic fetus during pregnancy. Fetal trophoblasts play a crucial role in circumventing a destructive maternal immune response in different ways. Fetal tissue can inhibit allogeneic immune responses by expressing IDO (that inhibits rapid proliferation of cells) (1, 2), FAS ligand (that can cause apoptosis of activated cells that express FAS) (3), and complement inhibitory proteins to prevent complement activation (4). These mechanisms can inhibit immune responses at the fetal-maternal interface in an Ag-nonspecific manner (5).

Trophoblasts do not express the classical HLA-A, HLA-B, HLA-DR, HLA-DQ, and HLA-DP molecules that are the main targets for alloreactive T cells in transplantation. However, trophoblasts do express HLA-C, HLA-E, and HLA-G molecules by which they can avoid NK cell-mediated cytotoxicity. HLA-G-expressing cells have been shown to induce regulatory T (Treg)[2] cells (6). In contrast, the highly polymorphic HLA-C can induce NK cell tolerance, but can also be a target for allogeneic T cells. Data from bone marrow transplantation patients have shown that a single HLA-C allele mismatch can elicit a cytotoxic T cell response (7) and is associated with a lower patient survival. In addition, HLA-E can decrease NK and CTL cytotoxicity (8), but has also been shown to exhibit alloantigenic properties that are indistinguishable from classical MHC class I molecules (9). Neutralization of possible CTLs with direct specificity for HLA-C, HLA-E, or indirectly presented minor histocompatibility Ags seems essential for the immunologic acceptance of the allogeneic fetus.

Maternal leukocytes present at the fetal-maternal interface include decidua-specific CD16^{-}CD56^{bright} NK cells and T cells, whereas B cells are virtually absent. Decidual NK cells have been shown to regulate trophoblast invasion by expression of NK cell receptors and the secretion of cytokines (10). Incompatibility of maternal killer Ig-like receptor (KIR) genotype and the fetal HLA-C allotype leads to increased risk of pregnancy complications like pre-eclampsia (11), suggesting that NK cells play a role in fetus-specific immune regulation.

Murine studies have shown that depletion of peripheral blood CD4^{+}CD25^{+} cells leads to gestation failure in allogeneic, but not in syngeneic pregnancy (12). These data suggest that T cells play a role in specific immune tolerance to fetal alloantigens in murine pregnancy. Recent studies have shown that high percentages of T cells are present in decidual tissue in human term pregnancy and that peripheral blood T cell subsets change during pregnancy (13–16). In addition, a significantly higher percentage of CD4^{+}CD25^{bright} T cells is present in decidual tissue compared with maternal PBL (mPBL) (13, 17). CD4^{+}CD25^{+} T cells are extensively studied by many groups for their regulatory capacities. Expression of CD25 is not exclusive for Treg cells. Effector T cells can also

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[2] Abbreviations used in this paper: Treg, T regulatory; cPBL, control PBL; d.basalis, decidua basalis; d.parietalis, decidua parietalis; mPBL, maternal PBL; S.I., suppression index; UCB, umbilical cord blood.

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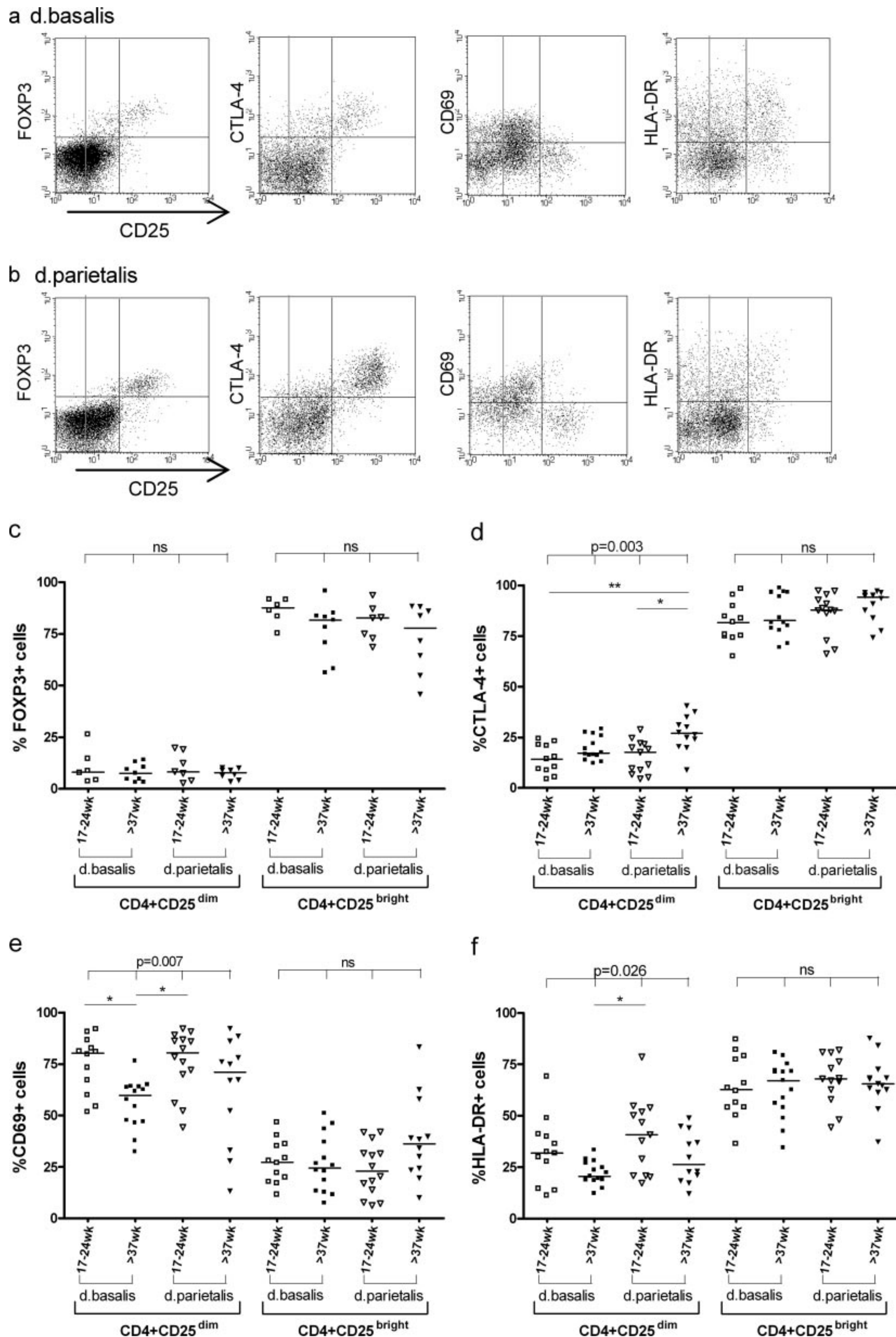

FIGURE 1. Characteristics of decidual $CD4^+CD25^{dim}$ and $CD4^+CD25^{bright}$ T cells. Representative dot plots of CD25 and intracellular FOXP3, intracellular CTLA-4, CD69, and HLA-DR expression in d.basalis (*a*) and d.parietalis (*b*) after term pregnancy. All plots are gated for $CD3^+CD4^+$ T cells within the lymphocyte gate. Percentage of $FOXP3^+$ (*c*), CTLA-4^+ (*d*), $CD69^+$ (*e*), and HLA-DR^+ (*f*) cells within $CD4^+CD25^{dim}$ or $CD4^+CD25^{bright}$ T cell fraction of d.basalis and d.parietalis in early pregnancy (17–24 wk) and after term (>37 wk) pregnancy. Lines indicate median percentages; *, $p < 0.05$; **, $p < 0.01$.

express high levels of CD25, whereas Treg cells can be found in the $CD25^-$ or $CD25^{dim}$ fraction (18, 19). Additional markers like CTLA-4 and FOXP3, and activation markers like HLA-DR and CD69 can help to distinguish effector from regulatory cells. However, conclusions based on phenotypic characterization remain controversial (20, 21). Until a specific marker for Treg

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cells is found, functional tests are required to identify and study Treg cells.

The aim of this study is to analyze phenotypic and functional properties of CD4$^+$CD25^{bright} T cells during pregnancy in tissue isolates from decidua basalis (d.basalis), the maternal part of the placenta at the implantation site connected to invading fetal trophoblasts; the decidua parietalis (d.parietalis), the maternal part of the membranes connected to the fetal trophoblasts of the chorion; and mPBL samples.

Materials and Methods

Blood and tissue samples

Paired samples of d.basalis, d.parietalis, heparinized mPBL, and heparinized umbilical cord blood (UCB) were obtained from healthy women after uncomplicated term pregnancy (gestational age range: 37–42 wk). Tissue samples were obtained after delivery by elective caesarean section or uncomplicated spontaneous vaginal delivery. Early pregnancy samples were obtained from healthy women undergoing surgical termination of pregnancy for social reasons (gestational age range: 17–23 wk). From the early decidua samples, in not all cases could paired d.basalis and d.parietalis be obtained. mPBL samples were obtained either directly before or directly after delivery or surgical curettage. UCB cells were obtained directly after delivery from the umbilical cord veins. Control PBL (cPBL) samples were obtained from healthy nonpregnant female volunteer donors (age range: 22–43 years). Tissue samples used for phenotypic analysis are partly similar to those described previously (13). Signed informed consent was obtained from all women, and the study received approval by the Leiden University Medical Centre Ethics Committee (P02-200).

Lymphocyte isolation

Lymphocyte isolation from decidua was done, as described previously (13). Shortly, d.basalis was macroscopically dissected from the maternal side of the placenta. D.parietalis was collected by removing the amnion and delicately scraping the d.parietalis from the chorion. The obtained tissue was washed thoroughly with PBS and thereafter finely minced between two scalpel blades in PBS. Decidual fragments were incubated with 0.2% collagenase I (Invitrogen Life Technologies) and 0.02% DNase I (Invitrogen Life Technologies) in RPMI 1640 medium, gently shaking in a waterbath at 37°C for 60 min, and thereafter washed once with RPMI 1640. The resultant suspensions were filtered through a 70-μm sieve (BD Discovery Labware) and washed once in RPMI 1640. For phenotypic analysis, the isolates were layered on Ficoll-Hypaque (Leiden University Medical Centre pharmacy) for density gradient centrifugation (20 min/800 $\times$ g). PBL and UCB samples were directly layered on a Ficoll-Hypaque gradient. Mononuclear cells were collected and washed twice with PBS containing 1% FCS, and all cells were fixed with 1% paraformaldehyde and stored at 4°C until cell staining and flow cytometric analysis. For functional analysis, the decidual lymphocyte isolates were layered on a Percoll gradient of (7.5 ml 1.08 g/ml; 12.5 ml 1.053 g/ml; 20 ml 1.034 g/ml) for density gradient centrifugation (30 min/800 $\times$ g) to minimize contaminating cell debris and nonlymphocyte cell types. Lymphocytes were isolated from the 1.08–1.053 g/ml interface. Comparison of the cell suspension obtained after Ficoll gradient and Percoll gradient isolation did not show any significant difference in composition of lymphocyte and T cell subsets (data not shown).

Flow cytometry

The following directly conjugated mouse anti-human mAb were used for four-color immunofluorescence surface staining: CD45 allophycocyanin, CD14 PE, CD25 PE, CD3 PerCP, CD4 allophycocyanin, CD69 FITC, and HLA-DR FITC (BD Biosciences), and used in concentrations according to manufacturer's instructions. For intracellular expression of CTLA-4, cells were stained for surface expression of CD3, CD4, and CD25; treated with permeabilizing solution buffer (containing: 0.1% saponine, 5% FCS, and 0.05% sodium-azide in PBS) for 10 min; and thereafter stained with anti-CTLA-4 allophycocyanin mAb (BD Biosciences). Intracellular expression of FOXP3 was determined using an allophycocyanin anti-human FOXP3 staining set (eBioscience), according to manufacturer's instructions. Flow cytometry was performed on a FACSCalibur using CellQuest-pro software (BD Biosciences), as described previously (13). The percentages of CD4$^+$CD25^{dim} and CD4$^+$CD25^{bright} T cells were calculated within the CD3$^+$CD4$^+$ cell fraction, and the percentages of FOXP3-, CTLA-4-, CD69-, and HLA-DR-positive cells were calculated within the CD3$^+$CD4$^+$CD25^{dim} or CD3$^+$CD4$^+$CD25^{bright} cell fractions. FACS analysis of all decidua and

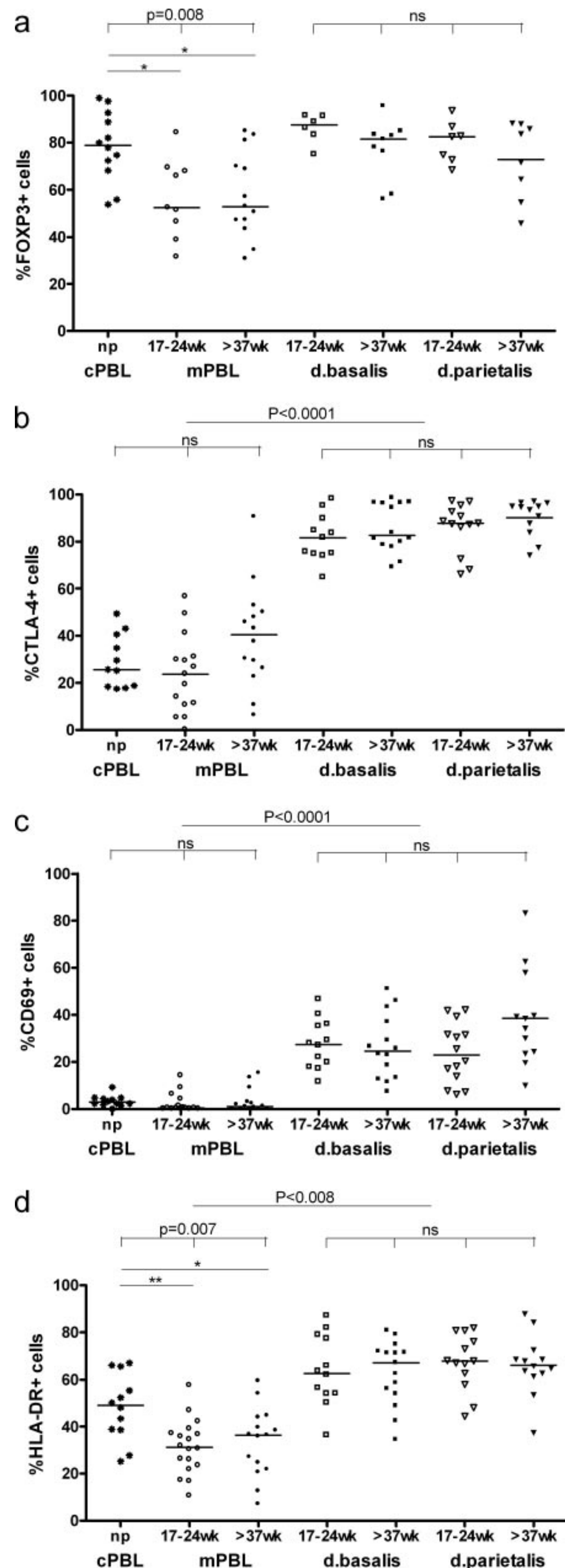

FIGURE 2. Characteristics of decidual and peripheral blood CD4$^+$CD25^{bright} cells. Percentage of FOXP3$^+$ (*a*), CTLA-4$^+$ (*b*), CD69$^+$ (*c*), and HLA-DR$^+$ (*d*) cells within CD4$^+$CD25^{bright} T cell fraction of nonpregnant (np) cPBL and mPBL, d.basalis, and d.parietalis in early (17–24 wk) and after term (>37 wk) pregnancy. Lines indicate median percentages; *, $p < 0.05$; **, $p < 0.01$.

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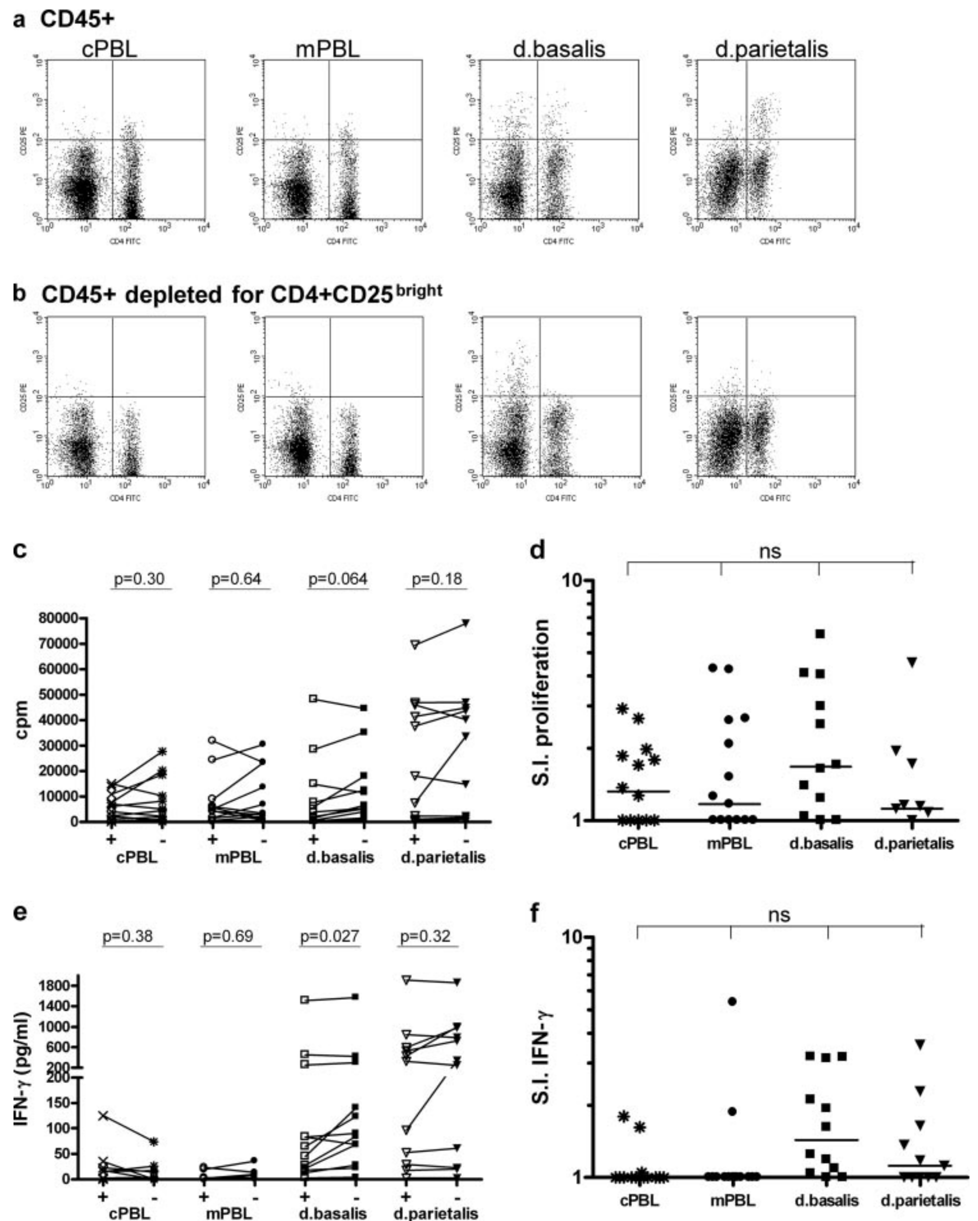

FIGURE 3. Function of CD4^{+} CD25^{bright} cells to OKT-3 stimulation. Representative dot plots of CD4 and CD25 expression within the CD45^{+} fraction (*a*) and the CD45^{+} fraction depleted for CD4^{+}CD25^{bright} cells (*b*) of cPBL, mPBL, d.basalis, and d.parietalis isolates after FACS sorting. All plots are gated for CD45^{+} cells within the lymphocyte gate. Proliferation (*c*) and IFN-γ production (*e*) of CD45^{+} cells (+) and CD45^{+} cells depleted for CD4^{+}CD25^{bright} cells (−) after OKT-3 stimulation. Isolates of cPBL, mPBL, d.basalis, and d.parietalis are shown. The S.I. of proliferation (*d*) and IFN-γ production (*f*) of all samples is depicted.

PBL samples was done using the same CellQuest-pro template; the fluorescence intensity to distinguish CD4^{+}CD25^{dim} and CD4^{+}CD25^{bright} cells was determined on decidual samples and exactly copied to PBL samples.

Functional assays

For functional analysis, the decidual and peripheral blood isolates were FACS sorted on a flow sorter ARIA (BD Biosciences) with DIVA software. Isolates were stained for surface CD4 FITC, CD25 PE, and CD45 allophycocyanin, and thereafter sorted for viable CD45^{+} cells or CD45^{+} cells without CD4^{+}CD25^{bright} cells. All cells were sorted within the lymphocyte gate set around the viable lymphocytes, avoiding granulocytes, macrophages, and other contaminating cell types. Cells were washed once in RPMI 1640 and thereafter incubated in RPMI 1640 supplemented with 2 mM L-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin (all obtained from Invitrogen Life Technologies), and 10% human serum in a round-bottom 96-well plate (Costar) at a density of 50,000 cells/well in triplicate. For anti-CD3 stimulation, wells were precoated with 10 μg/ml, 2 μg/ml OKT-3 (Orthoclone) or 5 μg/ml, 1 μg/ml UCHT-1 (BD Pharmingen) for 2 h at 37°C. For stimulation with UCB, 50,000 irradiated (3000 rad) UCB cells were added. All responders and stimulator cells were DNA typed for HLA-A, -B, -C, -DR, and -DQ. Cells were incubated at 37°C with 5% CO_2. At day 4, 50 μl of supernatant was collected and stored at −20°C until the time of analysis. Supernatants were analyzed with a Th1-Th2 Bio-plex premixed human cytokine panel Th1/Th2 (containing IL-2, IL-4, IL-5, IL-10, IL-13, GM-CSF, IFN-γ, and TNF-α; Bio-Rad), according to manufacturer's description. After the collection of the supernatants, proliferation was measured as [^{3}H]thymidine (1 μCi) incorporation for another 16 h by liquid scintillation spectroscopy using a betaplate (PerkinElmer-Wallac). Results were expressed as the median cpm for each triplicate culture. The suppression index (S.I.) (2) of CD4^{+}CD25^{bright} T cells is depicted as the ratio between paired proliferation (cpm) or cytokine production (pg/ml) of the CD45^{+} fraction depleted for CD4^{+}CD25^{bright} cells and the CD45^{+} fraction. All samples below the background of 700 cpm or 7 pg/ml IFN-γ are excluded to calculate a S.I., and samples with a negative S.I. are depicted as 0.

Immunohistochemistry

Paired d.basalis and d.parietalis isolates of early and term pregnancies were embedded in paraffin for immunohistochemical analysis. Serial 4-μm-thick tissue sections were deparaffinized using xylene and 100% ethanol and rehydrated with 70 and 50% ethanol. Endogenous peroxidase activity was blocked with methanol containing 0.3% H_2O_2. Ag retrieval was performed by microwaving the sections for 12 min in boiling citrate buffer (10 mMol/L (pH 6.0)). The tissue sections were incubated with the primary Ab diluted in PBS containing 1% BSA overnight in a moist chamber. Sections were washed three times and incubated with secondary Ab for 60 min in a

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moist chamber. Following three washes in PBS, the sections were embedded in Mowiol (Calbiochem). The Abs used are as follows: rabbit polyclonal CD4 (Santa Cruz Biotechnology); rabbit polyclonal CD3 (Abcam); goat anti-rabbit IgG Texas Red (Abcam); mouse monoclonal to FOXP3 (236A/E7; Abcam); and goat anti-mouse IgG1-FITC (BD Biosciences). The localization of CD4$^+$FOXP3$^+$ or CD3$^+$FOXP3$^+$ cells was determined using fluorescence microscopy.

Statistical analysis

To determine differences between >2 groups, a nonparametric Kruskal-Wallis one-way ANOVA was performed. If $p < 0.05$, a Dunn's multiple comparison posttest was performed to compare all pairs of columns. The Wilcoxon signed rank test was performed to determine differences between paired groups. The Mann-Whitney U test was used to determine differences between nonpaired groups. Values of $p < 0.05$ are considered to denote significant differences.

Results

Characterization of decidual CD4$^+$CD25^{dim} and CD4$^+$CD25^{bright} T cells

Consistent with a previous report by our group (13), we observed a significantly higher percentage of CD4$^+$CD25^{bright} T cells in all decidual samples compared with nonpregnant control PBL samples and mPBL samples. In addition, a significantly higher percentage of CD4$^+$CD25^{bright} T cells is observed in d.parietalis compared with d.basalis both in early (17–24 wk) and term pregnancy (>37 wk) (data not shown). To further characterize decidual CD4$^+$CD25^{dim} and CD4$^+$CD25^{bright} T cells, we performed flow cytometric analysis for the Treg markers FOXP3 and CTLA-4 (both intracellular), and surface expression of the T cell activation markers CD69 and HLA-DR. Representative FACS plots and the gating strategy for determining CD4$^+$CD25^{dim} and CD4$^+$CD25^{bright} T cells and FOXP3$^+$, CTLA-4$^+$, CD69$^+$, and HLA-DR$^+$ cells are shown in Fig. 1, *a* and *b*.

The decidual CD4$^+$CD25^{dim} and CD4$^+$CD25^{bright} T cell populations are two clearly distinctive cell populations. Decidual CD4$^+$CD25^{bright} T cells show a regulatory phenotype with high percentages of FOXP3$^+$, CTLA-4$^+$, and HLA-DR$^+$ cells and low percentages of CD69$^+$ cells. In contrast, the CD4$^+$CD25^{dim} T cell fraction of all decidual isolates shows an activated phenotype containing low percentages of FOXP3$^+$, CTLA-4$^+$, and HLA-DR$^+$ cells and high percentages of CD69$^+$ cells. The decidual CD4$^+$CD25^{bright} T cell population is a small, but homogeneous cell population with no significant differences in percentage of FOXP3$^+$, CTLA-4$^+$, CD69$^+$, and HLA-DR$^+$ cells between d.basalis and d.parietalis samples and no differences between early (17–24 wk) and term (>37 wk) pregnancy samples (Fig. 1, *c–f*). The decidual CD4$^+$CD25^{dim} T cell population contains minor differences in percentages of CTLA-4$^+$ cells and CD69$^+$ cells between d.basalis and d.parietalis samples and early (17–24 wk) and term (>37 wk) pregnancy samples (Fig. 1, *c–f*).

Different phenotype of CD4$^+$CD25^{bright} T cells in decidua compared with peripheral blood

To compare the phenotype of decidual and peripheral blood CD4$^+$CD25^{bright} T cells, peripheral blood samples from healthy nonpregnant female donors and the mPBL samples from early and term pregnancy were analyzed similar to the decidual isolates. All decidual CD4$^+$CD25^{bright} T cell fractions contain a significantly higher percentage of CTLA-4$^+$, CD69$^+$, and HLA-DR$^+$ cells compared with nonpregnant control PBL ($p < 0.0001$; $p < 0.0001$; $p < 0.008$, respectively), and mPBL both in early and term pregnancy (all p values <0.0001) (Fig. 2, *b–d*). In addition, a significantly higher percentage of FOXP3$^+$ cells is observed in decidual CD4$^+$CD25^{bright} T cells compared with CD4$^+$CD25^{bright} T cells from mPBL ($p < 0.0001$). However, no significant difference in percentage of FOXP3$^+$ cells in the decidual CD4$^+$CD25^{bright} T cell fractions compared with the CD4$^+$CD25^{bright} T cell fractions nonpregnant control PBL is observed (Fig. 2*a*).

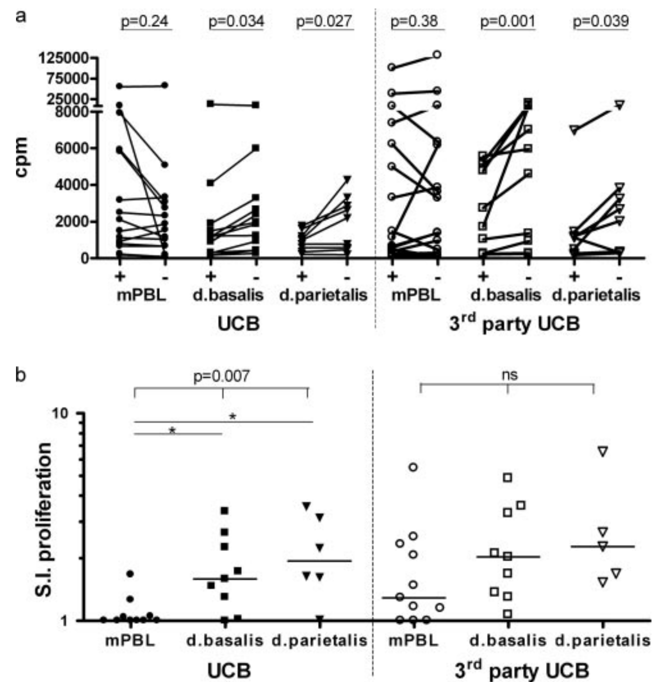

FIGURE 4. Fetus-specific and fetus-nonspecific suppression by decidual CD4$^+$CD25^{bright} T cells. *a*, Shows proliferation of CD45$^+$ cells (+) and CD45$^+$ cells depleted for CD4$^+$CD25^{bright} cells (−) after fetus-specific UCB (*left*) and third party UCB stimulation (*right*). Isolates of mPBL, d.basalis, and d.parietalis are shown. *b*, Shows the S.I. of proliferation of mPBL, d.basalis, and d.parietalis after fetus-specific UCB (*left*) and third party UCB stimulation (*right*) (*, $p < 0.05$; **, $p < 0.01$).

Comparison of the CD4$^+$CD25^{bright} T cell fraction from mPBL and nonpregnant control PBL shows a significantly lower percentage of FOXP3$^+$ in the CD4$^+$CD25^{bright} T cell fraction in mPBL in early (52%) and term (53%) pregnancy, compared with the CD4$^+$CD25^{bright} T cell fractions of nonpregnant controls (79%) ($p < 0.05$; $p < 0.05$; Fig. 2*a*). In addition, the CD4$^+$CD25^{bright} T cell fraction in mPBL in early (35%) and term (37%) pregnancy contains significantly less HLA-DR$^+$ cells compared with nonpregnant controls (50%) ($p < 0.01$; $p < 0.05$; Fig. 2*d*).

Functional analysis of CD4$^+$CD25^{bright} T cells

To examine the suppressive capacity of decidual and peripheral blood CD4$^+$CD25^{bright} T cells, we isolated a lymphocyte fraction containing all CD45$^+$ cells and a CD45$^+$ fraction depleted for CD4$^+$CD25^{bright} T cells by FACSort. Representative FACS plots and the gating strategy are shown in Fig. 3, *a* and *b*. Both fractions were stimulated with plate-bound OKT-3 (10 and 2 μg/ml) and plate-bound UCHT-1 (5 and 1 μg/ml) and examined for proliferation capacity by tritium incorporation, whereas the supernatants were examined for cytokine production by a Bio-plex bead array.

The proliferative capacity and IFN-γ production of peripheral blood isolates were not affected by depletion of the CD4$^+$CD25^{bright} cells using OKT-3 or UCHT-1 stimulation. In contrast, the d.basalis isolate shows a significant increase in IFN-γ production after depletion of the CD4$^+$CD25^{bright} cells ($p = 0.027$) and a slight, but not significant increase in proliferation ($p = 0.064$) using OKT-3 stimulation (Fig. 3, *c* and *e*). UCHT-1 stimulation induces high proliferative responses (range 61,000–240,000 cpm)

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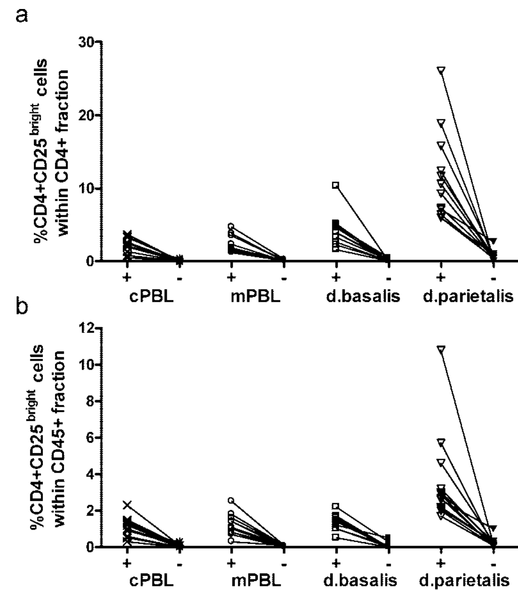
and IFN-γ production (range 175–6,400 pg/ml) in all decidual isolates. However, proliferation and IFN-γ production after UCHT-1 stimulation were not affected by depletion of CD4^{+}CD25^{bright} cells in all isolates. In d.parietalis, a group of high responders (proliferation $>$ 30,000 cpm and IFN-γ $>$ 400 pg/ml) and group of low responders (proliferation $<$ 10,000 cpm and IFN-γ $<$ 100 pg/ml) can be identified (Fig. 3, *c* and *e*). Both groups were checked for differences in clinical parameters (birth order, time of membrane rupture, maternal age, etc.) that could have led to an increased immune activation. There was no difference in any of these parameters, except for gender of the child: the high responders carried all female children (n = 5) and the low responders all male children, except for 1 female (n = 5 + 1). To compare the suppressive capacity of the four different isolates, a S.I. was determined, but no significant differences were observed with regard to proliferation (Fig. 3*d*) and IFN-γ production (Fig. 3*f*). Besides IFN-γ, all other cytokines (IL-2, IL-4, IL-5, IL-10, IL-13, GM-CSF, and TNF-α) were analyzed, but no significant differences in these cytokine concentrations were observed in the CD45^{+} fraction compared with the CD4^{+}CD25^{bright} depleted fraction.

Fetus-specific suppression capacity of CD4^{+}CD25^{bright} T cells

To determine whether there is a fetus-specific component in the suppressive capacity of mPBL and decidual CD4^{+}CD25^{bright} T cells, CD45^{+} cells and a CD45^{+} fraction depleted for CD4^{+}CD25^{bright} T cells were stimulated with UCB cells of the fetus and with a third party UCB. In both d.basalis and d.parietalis isolates, the depletion of CD4^{+}CD25^{bright} T cells leads to a significant increase in proliferation to UCB cells (p = 0.034; p = 0.027) and a third party UCB (p = 0.001; p = 0.039) (Fig. 4*a*). To compare the suppressive capacity of mPBL and the decidual isolates, a S.I. was determined. CD4^{+}CD25^{bright} T cells from d.basalis and d.parietalis contain a significantly higher suppressive capacity to regulate fetus-specific UCB cells compared with mPBL CD4^{+}CD25^{bright} T cells (p < 0.05; p < 0.0.05) (Fig. 4*b*). However, no difference in suppressive capacity between decidual and mPBL CD4^{+}CD25^{bright} T cells to third party UCB cells is observed (Fig. 4*b*). Interestingly, mPBL shows a reduced suppressive capacity to UCB of her own fetus (median SI = 1.0) compared with UCB of a third party fetus (median SI = 1.29) (p = 0.052) (data not shown). All mother-child combinations are haploidentical for HLA-A, -B, -C, -DR, and -DQ. No difference is observed between fully mismatched or haploidentical third party UCB stimulator cells using maternal and nonpregnant control responder cells. The capacity of mPBL to suppress third party UCB is similar to the capacity of nonpregnant controls to suppress UCB (data not shown).

Percentage of CD4^{+}CD25^{bright} T cells

To investigate whether the observed difference in suppressive capacity is caused by differences in percentages of CD4^{+}CD25^{bright} cells in the isolates, all fractions obtained after FACS sorting were reanalyzed on a FACSCalibur. The percentages of CD4^{+}CD25^{bright} T cells within the CD4^{+} T cell population and within the CD45^{+} populations were determined. In line with previous studies, d.basalis and d.parietalis lymphocyte isolates contain higher percentages of CD4^{+}CD25^{bright} T cells within the CD4^{+} T cell population compared with peripheral blood isolates (Fig. 5). Within the CD45^{+} fraction, the cPBL, mPBL, and d.basalis contain similar percentages of CD4^{+}CD25^{bright} T cells (median percentages: 1.0, 1.1, and 1.4%, respectively), resulting in Treg-lymphocyte ratio of ~1:100. D.parietalis contains a higher percentage



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%CD4+CD25 bright cells		CD45+	CD45+ depleted for CD4+CD25 bright
cPBL	within CD4+	2.1%	0.1%
	within CD45+	1.0%	0.1%
mPBL	within CD4+	1.8%	0.1%
	within CD45+	1.1%	0.1%
d.basalis	within CD4+	3.9%	0.1%
	within CD45+	1.4%	0.0%
d.parietalis	within CD4+	10.0%	0.6%
	within CD45+	2.8%	0.2%

FIGURE 5. Percentage of CD4^{+}CD25^{bright} T cells. Percentage of CD4^{+}CD25^{bright} T cells in CD45^{+} fraction (+) and CD45^{+} fraction depleted for CD4^{+}CD25^{bright} T cells (−) of cPBL, mPBL, d.basalis, and d.parietalis isolates after FACS sorting. Percentages of CD4^{+}CD25^{bright} T cells within the CD45^{+} T cell population (*a*) and within the CD4^{+} population (*b*) are depicted. *c*, Indicates the median percentages of CD4^{+}CD25^{bright} T cells of all samples.

of CD4^{+}CD25^{bright} T cells (2.8%) within the CD45^{+} fraction, resulting in a ratio of 1:36. No correlation between the Treg-lymphocyte ratios and the suppression index of all individual experiments was observed. The percentage of CD4^{+}CD25^{dim} T cells did not differ in the CD45^{+} fraction and the CD4^{+}CD25^{bright} depleted fraction (data not shown).

Localization of decidual CD3^{+}FOXP3^{+} cells at the fetal-maternal interface

In order to confirm the localization of CD3^{+} Treg cells in decidual tissue, we analyzed paraffin-embedded tissue sections of the placenta (containing d.basalis and villi) and the membranes (containing amnion, chorion, and d.parietalis) in early and term pregnancy. The sections were stained for CD4 in combination with FOXP3 or CD3 in combination with FOXP3. All sections show a preferential localization of CD4^{+}FOXP3^{+} and CD3^{+}FOXP3^{+} cells in maternal tissue (i.e., present in d.basalis, but not in villous tissue (Fig. 6*a*), and in d.parietalis, but not in chorion and amnion (Fig. 6*b*)). In addition, a high variation in numbers of CD3^{+}FOXP3^{-} and CD3^{+}FOXP3^{+} is observed between individual patients (Fig. 6, *b* and *c*).

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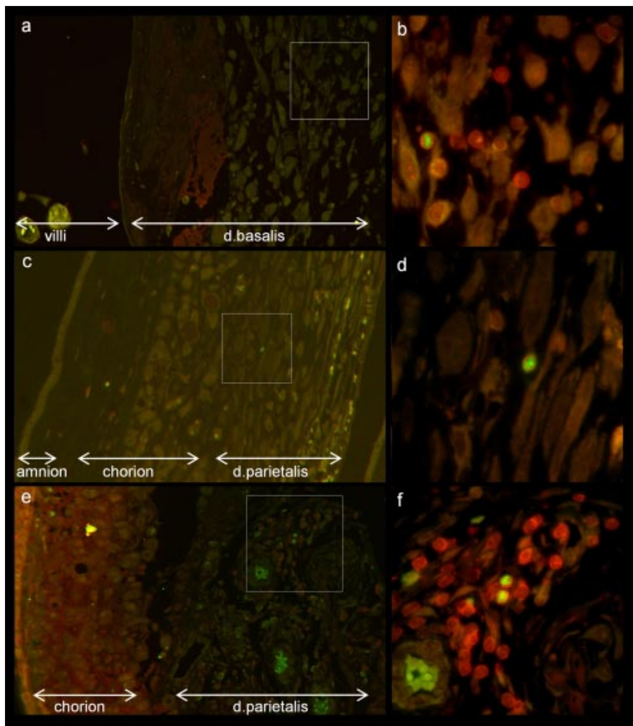

FIGURE 6. Localization of $CD3^+FOXP3^+$ cells at the fetal-maternal interface. Immunohistochemical staining of CD3-Texas Red and FOXP3-alexa488 in placenta sections (*a* and *b*) and sections of the membranes (*c–f*). *a*, Shows an overview of placental tissue containing villi and d.basalis; *b*, shows the localization of $CD3^+FOXP3^+$ and $CD3^+FOXP3^-$ cells in d.basalis. *c*, Shows an overview of membranes containing amnion, chorion, and d.parietalis tissue; *d*, shows the localization of a $CD3^+FOXP3^+$ cell in d.parietalis. *e*, Shows an overview of membranes from a second individual containing chorion and d.parietalis tissue; *f*, shows the localization of $CD3^+FOXP3^+$ and $CD3^+FOXP3^-$ cells in d.parietalis.

Discussion

In this study, we investigated the phenotypic and functional properties of decidual and peripheral blood $CD4^+CD25^{bright}$ T cells. Two clearly distinguished populations of $CD4^+CD25^{dim}$ and $CD4^+CD25^{bright}$ T cell subsets were found in all decidual isolates. $CD4^+CD25^{dim}$ T cells show an activated phenotype containing high percentages of $HLA\text{-}DR^+$ and $CD69^+$ cells and low percentages of $FOXP3^+$ and $CTLA\text{-}4^+$ cells. In contrast, decidual $CD4^+CD25^{bright}$ T cells show a regulatory phenotype containing high percentages of $FOXP3^+$, $CTLA\text{-}4^+$, and $HLA\text{-}DR^+$ cells. Decidual $CD4^+CD25^{bright}$ T cells are a homogeneous cell population with no significant differences in phenotype between d.basalis and d.parietalis isolates or between second and third trimester pregnancies. Decidual $CD4^+CD25^{bright}$ T cells show an increased expression of CTLA-4, HLA-DR, CD69, and CD25 compared with peripheral blood $CD4^+CD25^{bright}$ T cells. Understanding the functional significance of the phenotypic differences in peripheral and decidual $CD4^+CD25^{bright}$ Treg cells is limited by the lack of true Treg-specific surface markers and therefore the inability to define mechanisms of suppression. Identification of Treg cells based upon their phenotypic characterization is controversial (20, 21), and functional tests are required to identify Treg cells. The identification of novel Treg-specific markers CD39 and CD73 that are functionally involved in immunosuppressive activity in mice (22) is promising for future studies, but their relevance remains to be confirmed in the human system. In addition, mechanistic studies on FOXP3 function or signaling of immunoregulatory molecules such as TGF-β show the dynamics of Treg generation (23, 24) and may eventually lead to elucidation of the differences between peripheral and decidual $CD4^+CD25^{bright}$ T cells.

Many studies have shown that $CD4^+CD25^{bright}$ T cells can suppress specific and nonspecific immune responses in a dose-dependent manner. Similar results were obtained in functional assays, which differed with regard to experimental setup, effector cell populations (total $CD3^+$ cells, $CD4^+CD25^-$ T cells), Treg-T effector ratios, sources of APCs, and readout systems (proliferation, cytokines) (25, 26). The aim of our study was to compare the contribution of $CD4^+CD25^{bright}$ T cells in regulating maternal lymphocyte responses at the fetal-maternal interface and in mPBL. For this, we isolated the complete lymphocyte fraction and compared proliferation and cytokine responses of the total lymphocyte fraction with the $CD4^+CD25^{bright}$ depleted lymphocyte fraction. In contrast to other studies in which the modulating effect of an isolated subpopulation of responder cells is tested, we measured the potential of $CD4^+CD25^{bright}$ cells to suppress the reactivity of the different lymphocyte populations, including $CD4^+CD25^+$, $CD8^+$ T cells, and NK cells, present in the blood or in the decidua, which is compatible to the in vivo situation. In addition, we used CD3 stimulation and stimulation with UCB cells to determine whether there is a fetus-specific component in $CD4^+CD25^{bright}$ T cell-mediated suppression.

Upon CD3 stimulation, we found a variable increase in proliferation or IFN-γ production after depletion of $CD4^+CD25^{bright}$ T cells. In the d.basalis, a significant increase in IFN-γ production after depletion of $CD4^+CD25^{bright}$ T cells was observed in all individuals. In the d.parietalis, a group of high responders with a clear increase in proliferation and IFN-γ production after depletion of $CD4^+CD25^{bright}$ T cells was found next to a group of low responders without a clear regulatory capacity of the $CD4^+CD25^{bright}$ cells. Between these two groups, the gender of the child differed, the high responders being all female ($n = 5$) and the low responders all male except for 1 female ($n = 5 + 1$). The numbers in this group are too small to state significance, but in further studies this difference should be further elucidated. These data are suggestive for an individual variation in the contribution of $CD4^+CD25^{bright}$ T cells in the regulation of the local immune response.

In this study, we did not observe differences in the suppression capacity of PBL isolates and decidual lymphocyte isolates to CD3 stimulation using the OKT-3 and UCHT-1 clone. The type of suppression assay we used, lacking APCs and the low ratio $CD4^+CD25^{bright}$ cells that are depleted from the total lymphocyte isolate, might lead to a low sensitivity to detect regulation. It does, however, provide the best reflection of the in vivo activation status of all lymphocytes and capacity of Treg cells to regulate their response. Nevertheless, future experiments should elucidate possible differences in regulatory capacity of decidual and peripheral $CD4^+CD25^{bright}$ T cells by mixing Treg cells and lymphocytes in higher ratios and testing the influence of APCs.

The dynamics of immune regulation during pregnancy are shown by the fact that depletion of $CD4^+CD25^{bright}$ T cells from mPBL does not affect the immune response to her own child, whereas immune regulation to a third party UCB is comparable to nonpregnant controls. In addition, mPBL samples show a reduced percentage of $CD4^+CD25^{bright}FOXP3^+$ and $CD4^+CD25^{bright}HLA\text{-}DR^+$ cells compared with peripheral blood of nonpregnant controls. In contrast, decidual tissue contains a high proportion of $CD4^+CD25^{bright}$ T cells with a regulatory phenotype and despite the individual variation between the patients; the decidual $CD4^+CD25^{bright}$ T cells contain the capacity to regulate fetus-specific

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and fetus-nonspecific responses. These data suggest that fetus-specific Treg cells are specifically recruited from the periphery to the fetal-maternal interface.

A recent study examining the dynamics of $CD4^+CD25^{bright}$ T cells during the menstrual cycle has demonstrated an expansion of $CD4^+CD25^{bright}FOXP3^+$ T cells just before ovulation (27). In addition, reduced numbers of Treg cells and a diminished suppressive capacity of these cells were observed in women with recurrent spontaneous abortions (17). Besides the impairment of expansion of functional Treg populations, defects in recruitment of $CD4^+CD25^{bright}$ Treg cells to the fetal-maternal interface may play a role in development of pathology during pregnancy.

The leukocyte composition of decidual isolates is highly variable among individuals (data not shown). Analysis of 14 uncomplicated term deliveries show an average T cell percentage of 51 ± 13% in d.basalis, 64 ± 11% in d.parietalis, and 71 ± 11% in mPBL (all calculated within the $CD45^+$ lymphocyte fraction), compared with 75 ± 3% in peripheral blood of nonpregnant controls. In addition, there is high variation in percentage of $CD4^+CD25^{bright}$ T cells in d.basalis and d.parietalis isolates (13). We did not find a correlation between the percentage of depleted $CD4^+CD25^{bright}$ T cells and the observed suppression capacity. However, the variation in suppression capacity between the samples might be due to a different leukocyte composition of the isolates. Decidual T cells comprise a very heterogeneous subset of T cells containing $CD4^+$ and $CD8^+$ cells with highly activated phenotypes as well as cells with a merely regulatory phenotype (13, 16). The activated decidual T cells might be more difficult to suppress in comparison with peripheral blood T cells, resulting in similar suppression indexes. The decidual isolates also contain variable percentages of decidual NK cells, and although studies have shown that $CD4^+CD25^+$ T cells can inhibit NK cell functions (28), future studies should examine the potential inhibitory effect of $CD4^+CD25^{bright}$ T cells on decidual NK cells.

Based on the high variation between lymphocyte properties in individual pregnancies, including lymphocyte gain, lymphocyte composition, and the variable contribution of $CD4^+CD25^{bright}$ T cells to suppress decidual lymphocyte responses, we hypothesize that each pregnancy generates a unique combination of regulatory mechanisms to result in a successful pregnancy. These regulatory mechanisms can include nonspecific suppression mechanisms mediated by the expression of IDO, FAS, complement inhibitor proteins, or more specific mechanisms mediated by HLA-expression patterns (1–5), NK cell-trophoblast interactions (10, 11), decidual macrophages, or Treg cells (1, 13, 14). Maternal genotype (such as HLA genotype, killer Ig-like receptor genotype, or cytokine polymorphisms), or maternal history (regarding birth order, infection history) and the combination of fetal HLA matches and mismatches may determine which regulatory mechanisms are most predominant.

The mechanisms by which Treg cells can inhibit fetus-specific responses at the fetal-maternal interface remain to be elucidated. Examining the functional differences between decidual and peripheral blood $CD4^+CD25^{bright}$ T cells might identify factors that can induce $CD4^+CD25^{bright}$ Treg cells at the fetal-maternal interface and may help to understand conditions of placental pathology in which Treg cells are reduced (17, 29). In this study, we demonstrate that fetus-specific Treg cells are absent in mPBL at term pregnancy. In addition, we demonstrate that decidual $CD4^+CD25^{bright}$ T cells suppress fetus-specific and fetus-nonspecific responses. Our data suggest a preferential recruitment of fetus-specific Treg cells from mPBL to the fetal-maternal interface and suggest that $CD4^+CD25^{bright}$ T cells contribute to the regulation of fetus-specific responses in human decidua.

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Disclosures

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