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**Systemic reduction of functionally suppressive CD4<sup>dim</sup>CD25<sup>high</sup>Foxp3<sup>+</sup> Tregs in human second trimester pregnancy is induced by progesterone and 17 $\beta$ -estradiol**

Running title: Tregs in human pregnancy

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**Key words:** Human, T cells, Cytokines, Reproductive Immunology

## Abstract

CD4<sup>+</sup>CD25<sup>high</sup> regulatory T cells (Tregs) are implicated in maintenance of murine pregnancy. However, reports regarding circulating Treg frequencies in human pregnancy are inconsistent and the functionality and phenotype of these cells in pregnancy have not been clarified. The aim was to determine the frequency, phenotype and function of circulating Tregs in second trimester human pregnancy and the influence of progesterone and 17 $\beta$ -estradiol on Treg phenotype and frequency. Based on expression of Foxp3, CD127 and HLA-DR, as determined by multi-color flow cytometry, we defined a proper CD4<sup>dim</sup>CD25<sup>high</sup> Treg population and showed, in contrast to most previous reports, that this population was reduced in second trimester pregnancy. Unexpectedly, Foxp3 expression was decreased in the Treg, as well as in the CD4<sup>+</sup> population. These changes could be replicated in an *in vitro* system resembling the pregnancy hormonal milieu, where 17 $\beta$ -estradiol, and in particular progesterone, induced, in line with the pregnancy situation, a reduction of CD4<sup>dim</sup>CD25<sup>high</sup>Foxp3<sup>+</sup> cells in PBMC from non-pregnant women. By co-culturing FACS-sorted Tregs and autologous CD4<sup>+</sup>CD25<sup>-</sup> responder cells, we showed that Tregs from pregnant women still displayed the same suppressive capacity as non-pregnant women in terms of suppressing IL-2, TNF- $\alpha$  and IFN- $\gamma$  secretion from responder cells while efficiently producing IL-4 and IL-10. Our findings support the view of hormones, particularly progesterone, as critical regulators of Tregs in pregnancy. Further, we suggest that in the light of the results of this study, early data on circulating Treg frequencies in pregnancy need re-evaluation.

## Introduction

Pregnancy is a state of partial tolerance since, during a successful pregnancy, the maternal immune system is aware of, and actively tolerates, the semi-allogenic fetus without dramatically compromising the maternal defence against infections. CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells (Tregs), first discovered as important protectors of autoimmune diseases in mice (1), have gained vast attention as key players in human tolerance.

Human Tregs are found within the CD4<sup>+</sup> subset expressing the highest level of the IL-2 receptor  $\alpha$ -chain CD25 (2), hence termed CD4<sup>+</sup>CD25<sup>high</sup> or CD4<sup>+</sup>CD25<sup>bright</sup> Tregs. However, the CD4<sup>+</sup>CD25<sup>high</sup> population is neither functionally nor phenotypically homogenous, but contains both suppressor and effector cells, highlighting the need for molecular markers that can accurately define suppressive Tregs. At present, the transcription factor Forkhead box p3 (Foxp3), involved in Treg lineage commitment (3, 4), is the best marker for Tregs. However, even though Foxp3 expression correlates with suppressive function in freshly isolated Tregs, Foxp3 can be up-regulated following *in vitro* activation of non-Tregs (5-7) and several factors such as hormones can influence Foxp3 expression (8). Recently, the IL-7 receptor CD127 was shown to be negatively regulated by Foxp3, and low CD127 expression (CD127<sup>low</sup>), correlating with suppressive function, was suggested a surrogate surface marker for Foxp3 (9, 10). Other molecules expressed by Tregs are HLA-DR and CTLA-4, both suggested to be involved in Treg suppression (11, 12).

Tregs have been implicated in the successful maintenance of pregnancy in mice, where Tregs increase during normal pregnancy and their absence leads to gestational failure (13, 14). In a murine abortion model, Tregs adoptively transferred from normal pregnant mice induce a fetal-protective microenvironment and provide protection against aggressive anti-fetal immune reactions (14, 15). The cause of the Treg-protective mechanisms and Treg expansion

seen during murine pregnancy has been ascribed pregnancy hormones (13), foremost estrogens (16), but also fetal alloantigens (14, 17).

In line with these murine data, we have shown that during normal human pregnancy, circulating Tregs suppress anti-fetal T<sub>H</sub>1 and T<sub>H</sub>2 like reactions as demonstrated by an *in vitro* Treg mixed leukocyte culture (MLC) - ELISPOT assay (18). However, the importance of Tregs in maintenance of human pregnancy *in vivo* is far from settled. Studies in humans have suggested that there is an increase in the CD4<sup>+</sup>CD25<sup>high</sup> population during normal pregnancy, most apparently at the fetal-maternal interface (19-21) but also in the circulation (19, 21-23). As in the murine system, estrogen seems to drive this suggested expansion (24), whereas the role of other pregnancy hormones such as progesterone, is unknown. Further, it remains obscure if the previous observations reflect an increase of actual suppressive Tregs since more specific markers such as Foxp3 have not been thoroughly investigated previously. As pregnancy could be considered a state of controlled immune activation (25, 26), possibly due to exposure and awareness of fetal antigens, the CD4<sup>+</sup>CD25<sup>high</sup> expansion seen during pregnancy might reflect an increase of activated CD4<sup>+</sup> T cells. Thus, in such settings, Tregs should be better characterised by proteins such as Foxp3, CTLA-4, HLA-DR and the recently described marker CD127<sup>low</sup>.

In this study, we aimed at determining the frequency, phenotype and function of stringently defined circulating Tregs in PBMC from healthy pregnant women, non-pregnant women and *in vitro* 17 $\beta$ -estradiol/progesterone stimulated PBMC of non-pregnant women. In contrast to most previous studies, only characterizing Tregs by CD4 and CD25 expression, we demonstrate a systemic reduction, caused mainly by progesterone but also by 17 $\beta$ -estradiol, of functionally suppressive CD4<sup>dim</sup>CD25<sup>high</sup>Foxp3<sup>+</sup> Tregs in human second trimester pregnancy.

## **Subjects and Methods**

### **Subjects**

Thirty-eight healthy pregnant women with no signs of pregnancy complications at inclusion (pregnancy week 24-28) visiting the maternity outpatient care unit in Linköping (Kvinnohälsan), were asked to participate in the study. Obstetrical history of these women is given in table I. Twenty-five women were pregnant for the first time and thirteen had experienced at least one previous pregnancy. Five women had previously given birth at least once. Excluding women with previous pregnancy did not affect the statistical results.

Seventy-one non-pregnant women (age 19-36 years, median age 26), who were blood donors or staff at Linköping University Hospital, served as control subjects. There were no statistical age differences between pregnant and non-pregnant women. In the functional *in vitro* assays, none of the non-pregnant women (n=27) were using hormonal contraceptives. In the *in vitro* suppression assay, five of the women were in proliferative phase, six in secretory phase and three women were on day 15 in their menstrual cycle according to questionnaires. No such information was available from the other non-pregnant women. Informed consent was obtained from all participants. The study was approved by the Local Ethics Committee at Linköping University, Sweden.

### **PBMC preparation**

Whole blood was obtained in EDTA (for flow cytometry) or sodium-heparin (for functional assays) vacutainer tubes. PBMC were separated within one hour on Lymphoprep (Axis-Shield, Oslo, Norway) according to the manufacturer's instructions followed by washing in Hank's balanced salt solution (HBSS; Gibco BRL, Paisley, Scotland; UK). For direct culturing, cells were resuspended in T cell culture medium (TCM) consisting of Iscove's

modified Dulbecco's medium (IMDM; Gibco BRL) supplemented with L-glutamine (292 mg/mL; Sigma Aldrich, Stockholm, Sweden), sodium bicarbonate (3,024 g/L; Sigma), penicillin (50 IE/mL), streptomycin (50 µg/mL) (Cambrex, New Jersey, USA), 100x non-essential amino acids (10 mL/L; Gibco BRL) and 5% heat inactivated fetal bovine serum (FBS; Sigma). For flow cytometry, cells were resuspended in PBS pH 7.4 (Medicago AB, Uppsala, Sweden) supplemented with 0.1 % or 2% heat inactivated FBS (Sigma). For MACS separation, cells were resuspended in PBS with 2 mM EDTA (Sigma) and 2% FBS. Alternatively, PBMC were lysed in RNeasy RLT lysing buffer (Qiagen, West Sussex, UK) and frozen at -80°C until RNA extraction.

### **RNA extraction and reverse transcriptase real-time PCR for quantification of FOXP3 mRNA expression**

Expression of Foxp3 mRNA was analyzed in PBMC from pregnant (n=13) and non-pregnant women (n=25). Total RNA was extracted using the RNeasy minikit (Qiagen) according to the manufacturer's instructions. Approximately 200 ng of RNA was converted to cDNA in 20µL reactions using the cDNA high-capacity archive kit (Applied Biosystems, Foster City, CA, USA) with RNase inhibitor (Applied Biosystems) according to the manufacturer's instructions. Reverse transcription was performed on a Mastercycler ep Thermal cycler (Eppendorf AG, Hamburg, Germany). For real-time PCR, 1 µL of cDNA was mixed with TaqMan Universal Mastermix (Applied Biosystems) together with primers and probe for Foxp3 or 18S rRNA, respectively (table II). cDNA was amplified according to the TaqMan standard protocol as described by the manufacturer. Reactions were performed using the 7500 Real-Time PCR System (Applied Biosystems). Expression of 18S rRNA was used for normalization of RNA content in all samples. The absence of genomic DNA amplification was controlled by amplifying one sample of RNA. Data were analyzed with the 7300 system

SDS software v 1.3.1 (Applied Biosystems). Quantification was performed using the standard curve method. All samples were analyzed in duplicates and the variation limit between the duplicates was set to <15%.

#### **Four-color flow cytometry**

PBMC from pregnant (n=14) and non-pregnant (n=34) women were analyzed using four-color flow cytometry to determine the frequency of CD4<sup>+</sup>CD25<sup>high</sup> cells and the expression of Foxp3, CTLA-4 and CD27. One million PBMC were incubated with mouse isotype controls (IgG1-FITC, IgG1-PE, IgG1-PerCP and IgG1-APC; all clones X40) (BD Biosciences, Franklin Lakes, NJ, USA) or mouse anti-human CD25-APC (clone 2A3), CD4-PerCP (clone SK3) and CD27-FITC (clone M-T271) (BD Biosciences) for 30 minutes at 4°C in darkness. Cells were washed in PBS 0.1% FBS by centrifugation at 500g for 5 minutes, followed by adding Fixation/Permeabilisation buffer (eBioscience, San Diego, CA, USA) and incubating as described above. Cells were washed twice in Permeabilisation buffer (eBioscience) by centrifugation at 500g for 5 minutes. Antibodies directed against intracellular CTLA-4 (CTLA-4<sub>i.c.</sub>; clone BNI3) (BD Biosciences) or Foxp3 (clone PCH101) (eBioscience) or isotype controls (BD Biosciences) were added and cells were incubated as above. Cells were washed once as described above and resuspended in PBS 0.1% FBS. The absence of CTLA-4 surface expression was confirmed on two separate occasions and this is in agreement with others (27). One hundred thousand lymphocytes were collected and analyzed using FACSCalibur and the CellquestPro software (BD Biosciences). The Foxp3 antibody clone PCH101 binds the amino-terminus of the Foxp3 protein and has been shown to recognise both isoforms of the Foxp3 protein (7).

### **Six-color flow cytometry**

PBMC from pregnant (n=10) and non-pregnant (n=10) women were analyzed by six-color flow cytometry to obtain a more detailed phenotype analysis. In addition,  $17\beta$ -estradiol and progesterone stimulated PBMC (see below) were analyzed this way. Cells were labelled with isotype controls, as described above, or mouse anti-human CD3-APC-Cy7 (clone SK7), CD4-PerCP, CD25-APC, CD45RA-FITC (clone L48), CD45R0-PE-Cy7 (clone UCHL1), CD127-PE (clone hIL-7R-M21), CD69-PE-Cy7 (clone FN50) and HLA-DR-FITC (clone L243). This was followed by permeabilisation/fixation and staining of intracellular Foxp3 protein as described above. One hundred thousand lymphocytes were collected and analyzed using FACSCanto II (BD Biosciences) and the FACSDiva software (version 5.0.1; BD Biosciences). Absolute leukocyte (CD45), T lymphocyte (CD3) and T helper cell counts (CD4) in 50 $\mu$ L of EDTA whole blood were determined by using TruCount tubes (BD Biosciences) as described by the manufacturer.

### **Flow cytometric gating and analysis**

All gating analysis was performed in a blinded manner, i.e. the evaluator did not know the origin of the sample. Cells were gated for analysis of lymphocytes by side/forward scatter, and in six-color flow cytometry, gating for analysis of T cells was also based on CD3 expression. Gates for expression of CD25 in the CD4 population ( $CD4^+CD25^+$  or  $CD4^+CD25^-$ ) were set according to isotype controls. The  $CD25^{high}$  gate was adjusted to contain  $CD4^+$  cells that expressed higher levels of CD25 than the discrete population of  $CD4^-$  cells that expressed CD25 (28, 29). This, and the development of other gating strategies, is further described in the results section. To avoid the possible errors introduced when subjectively setting any gate for  $CD25^{high}$  expression, the 0.5% of  $CD4^+$  cells expressing the highest levels of CD25 were also evaluated. These cells are referred to as “0.5%  $CD4^+CD25^{highest}$ ” cells. Mean fluorescence

intensity (MFI) was evaluated by dividing the geometric MFI (gMFI) for Foxp3<sup>+</sup> cells with the gMFI for Foxp3<sup>-</sup> isotype controls, to correct for the instrumental day-to-day variations in fluorescence intensity measurements.

### **Stimulation of peripheral blood mononuclear cells (PBMC) with 17 $\beta$ -estradiol and progesterone**

6-well plates (Costar, Cambridge, MA, USA) were coated with 0.005  $\mu$ g/mL mouse anti-human CD3 antibody (clone UCHT1; AbD Serotec, Oxford, UK) for 2 hours at 37°C followed by washing the wells with PBS. The chosen anti-CD3 antibody concentration was based on titration experiments where 0.005  $\mu$ g/mL anti-CD3 antibody caused a low grade activation of the CD4<sup>+</sup> cells with slight elevation of CD69 and CD25 expression. PBMC, isolated from non-pregnant women (n=13), at a final concentration of 10<sup>6</sup> PBMC/mL, were cultured in uncoated or anti-CD3 antibody coated wells with 10nM, 100nM or 10  $\mu$ M 17 $\beta$ -estradiol (water soluble E; Sigma Aldrich) and/or 200 nM, 2  $\mu$ M or 200  $\mu$ M progesterone (water soluble P; Sigma Aldrich) in TCM for 3 days at 37°C, 5% CO<sub>2</sub> in a humidified atmosphere. After incubation, cells were stained for six-color flow cytometry analysis on FACSCanto II as described above.

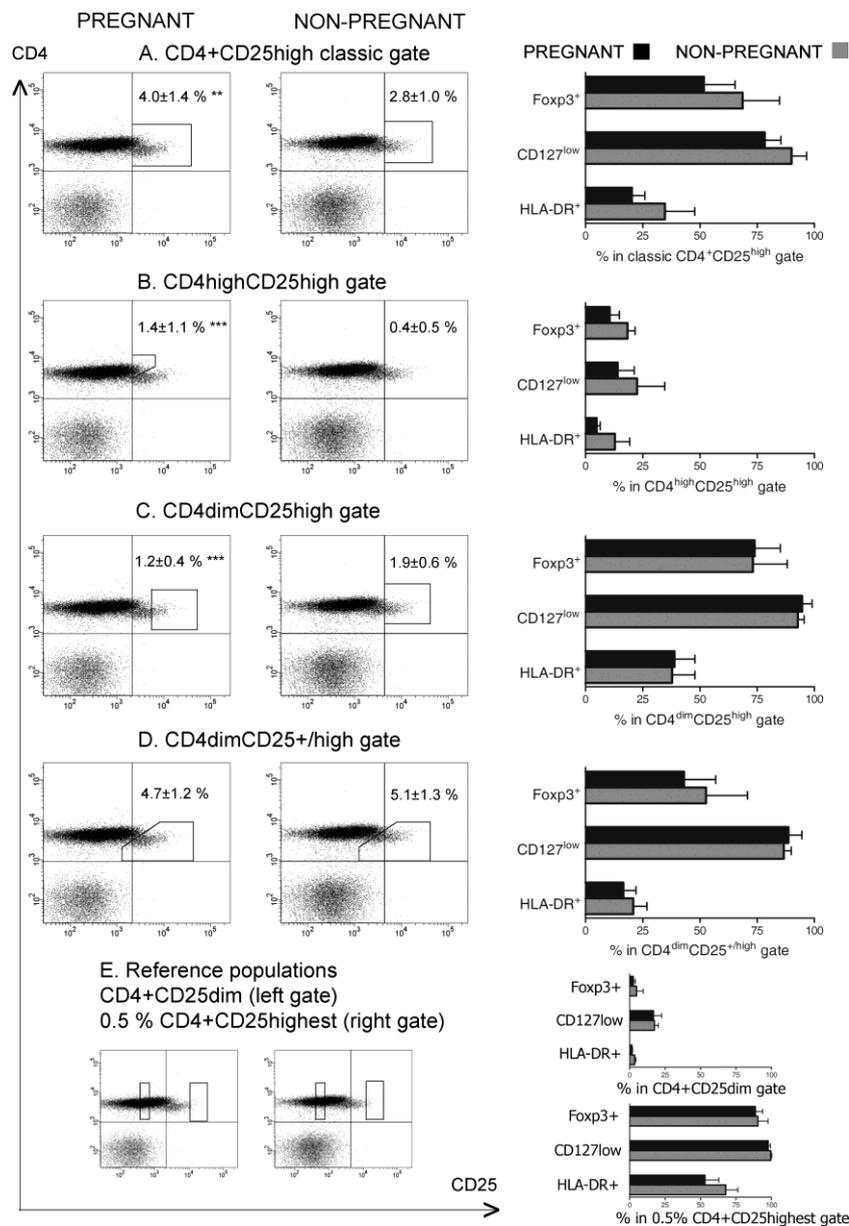
### **Functional suppression assay – MACS-FACS-sorting and culturing conditions**

The CD4<sup>+</sup> T cell isolation kit II (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) was used for negative selection of untouched CD4<sup>+</sup> cells from PBMC separated from pregnant (n=14) and non-pregnant women (n=14). CD4<sup>+</sup> selection was performed according to the manufacturer's description using MS columns and a miniMACS separator (Miltenyi Biotec). The MACS sorted CD4<sup>+</sup> cells were then labelled with mouse anti-human CD4-FITC (clone MT466; Miltenyi Biotec) and mouse anti-human CD25-APC (BD Biosciences). For analysis,

a portion of cells was also labelled with mouse anti-human CD127-PE (BD Biosciences). Sorting of CD4<sup>+</sup>CD25<sup>-</sup> responder cells and CD4<sup>dim</sup>CD25<sup>high</sup> Tregs (see below and fig 1c for gating) was performed on a FACSAria cell sorter (BD Biosciences) equipped with a 100µm nozzle. Sorted populations were collected in TCM and typically showed purities above 99% upon reanalysis. 96-well plates (BD Biosciences, Le Pont De Claix, France) were coated with 1 or 5µg/mL anti-CD3 antibody (clone UCHT1; AbD Serotec) and 5µg/mL rat anti-human CD28 (clone YTH913.12; AbD Serotec) for 24 hours at 4°C followed by washing in PBS. CD4<sup>+</sup>CD25<sup>-</sup> responder cells were plated at  $2.5 \times 10^4$  cells/well alone or in co-culture with CD4<sup>dim</sup>CD25<sup>high</sup> Tregs at ratios of 1:1, 2:1 or 4:1 in singlet or duplicate cultures and cultured for 91-93 hours at 37°C and 5% CO<sub>2</sub> in a humidified atmosphere before harvesting the supernatants. The ability of CD4<sup>dim</sup>CD25<sup>high</sup> Tregs to suppress cytokine secretion from CD4<sup>+</sup>CD25<sup>-</sup> responder cells was calculated as a suppressive index (SI) according to:  $(1 - (\text{secretion in co-culture} / \text{secretion from CD4}^+ \text{CD25}^- \text{ cells alone})) \times 100$ .

### **Multiplex bead array analysis of IL-2, IL-4, IL-10, TNF- $\alpha$ and IFN- $\gamma$**

Supernatants were analyzed by LINCOplex human cytokine kit according to the manufacturer's instructions using the Luminex 100 instrument (Luminex Corporation, Austin, Texas, USA). STarStation software (v 2.3, Applied Cytometry Systems, Sheffield, UK) was used for acquisition and analysis of data. The range of the standard curves was 0.13-10 000 pg/ml with a dilution factor of 5. The lowest (detection limit) and highest standard concentrations used for each cytokine was adjusted according to the standard curve fitting of the standard concentrations after mathematical interpolation. Values below the detection limit were given half the value of the detection limit.



**Figure 1.** Different gating strategies for gating of Tregs (left column; pregnant  $n=14$ , non-pregnant 34) and the expression of Treg associated markers Foxp3, CD127<sup>low</sup> and HLA-DR within these gates (right column; pregnant  $n=10$ , non-pregnant 10) in cells from pregnant and non-pregnant women. A. The classical CD4<sup>+</sup>CD25<sup>high</sup> gate was adjusted to contain CD4<sup>+</sup> cells that express higher levels of CD25 than CD4<sup>-</sup> cells. B. Pregnant women show a distinctly scattered population of cells with high CD4 expression; CD4<sup>high</sup>CD25<sup>high</sup> which show low prevalence of Foxp3<sup>+</sup>, CD127<sup>low</sup> and HLA-DR<sup>+</sup> cells. C. To avoid CD4<sup>high</sup>CD25<sup>high</sup> non-Tregs, a gate was set to include the CD25<sup>high</sup> cells with lower expression of CD4; CD4<sup>dim</sup>CD25<sup>high</sup>. D. Distinctly scattered cells with lowered CD4 expression; CD4<sup>dim</sup>CD25<sup>+/high</sup>. E. Reference populations: Activated CD4<sup>+</sup> cells; CD4<sup>+</sup>CD25<sup>dim</sup> showing a non-Treg-phenotype, and regulatory T cells; 0.5% CD4<sup>+</sup>CD25<sup>highest</sup> cells showing a clear-cut Treg-phenotype. See text for further explanation.

All numbers and bars are given as means  $\pm$  SD. \*\*  $p \leq 0.01$ , \*\*\*  $p \leq 0.001$

## Statistics

The statistical guidance resource at Linköping University was consulted for the statistical analyzes. Due to multiple comparisons and the risk of mass significances, the significance level was set to 1%, i.e.  $p \leq 0.01$  (sometimes depicted as \*\*) was considered statistically significant and  $p \leq 0.05$  (sometimes depicted as \*) was regarded as a statistical tendency. Results from the flow cytometric analyzes were analyzed using Student's unpaired t-test and presented as mean  $\pm$  SD. Data on cytokines did not follow Gaussian distribution and were therefore analyzed using Wilcoxon signed rank test or Mann-Whitney U test and presented as medians and interquartile range (25<sup>th</sup> and 75<sup>th</sup> percentile values). Data on cytokines were also logarithmically transformed, to obtain Gaussian distribution, and analyzed using parametrical statistical methods. Since this did not affect the results, data was kept in linear mode and analyzed non-parametrically as described above. The coefficient of variation (CV) was expressed as percentage by calculating  $(SD/mean) \times 100$ . All statistical analyzes were performed using the GraphPad Prism version 4 software (GraphPad Software Inc., San Diego, CA, USA).

## Results

### **Determination of an optimal gating strategy for CD4<sup>+</sup>CD25<sup>high</sup> cells during pregnancy; The CD25<sup>high</sup> cells with low CD4 expression (CD4<sup>dim</sup>CD25<sup>high</sup>) show the most pronounced Treg phenotype**

The optimal flow cytometric gating strategy for CD25<sup>high</sup> cells was investigated using four-color flow cytometry, in conjunction with six-color-flow cytometry for analysis of the Treg markers Foxp3, CD127<sup>low</sup> and HLA-DR. CD4<sup>+</sup>CD25<sup>high</sup> cells were first gated according to the classical CD4<sup>+</sup>CD25<sup>high</sup> gate; i.e. adjusted to contain CD4<sup>+</sup> cells that expressed higher levels of CD25 than the discrete population of CD4<sup>-</sup> cells that express CD25 (fig 1a) as described previously by others (28, 29). Using this gating strategy, pregnant women displayed an increased frequency of CD4<sup>+</sup>CD25<sup>high</sup> cells of CD4<sup>+</sup> cells (fig 1a). However, it was noted that within the classical CD4<sup>+</sup>CD25<sup>high</sup> gate, the pregnant women showed a population of distinctly scattered cells with high CD4 expression (CD4<sup>high</sup>). This CD4<sup>high</sup>CD25<sup>high</sup> population was expanded in pregnant women (fig 1b) while almost absent in non-pregnant women (<0.5% of CD4<sup>+</sup> in 35 of total 44 controls). Importantly, detailed subpopulation analysis showed that the CD4<sup>high</sup>CD25<sup>high</sup> population contained few cells expressing the Treg markers Foxp3 and CD127<sup>low</sup> as well as HLA-DR (fig 1b right panel). Thus, the CD4<sup>high</sup>CD25<sup>high</sup> population was more similar to activated CD4<sup>+</sup>CD25<sup>dim</sup> cells than the 0.5% CD4<sup>+</sup>CD25<sup>highest</sup> cells regarding the expression of Foxp3, CD127 and HLA-DR (fig 1b and 1e, right panels), and also with regard to high cytokine secretion and lack of suppressive activity (see further below). To avoid inclusion of these apparent non-Tregs, a gate was set to include CD25<sup>high</sup> cells with a lower expression of CD4; i.e. CD4<sup>dim</sup>CD25<sup>high</sup> (fig 1c). This CD4<sup>dim</sup>CD25<sup>high</sup> population showed a high resemblance with the 0.5% CD4<sup>+</sup>CD25<sup>highest</sup> cells,

with high prevalence of Foxp3<sup>+</sup>, CD127<sup>low</sup> and HLA-DR<sup>+</sup> cells (fig 1c and 1e, right panels). Therefore, the CD4<sup>dim</sup>CD25<sup>high</sup> was considered the optimal definition of Tregs. To our surprise, the CD4<sup>dim</sup>CD25<sup>high</sup> population was reduced in size (in % of CD4<sup>+</sup> cells) in pregnant as compared to non-pregnant women (fig 1c). We also considered a distinctly scattered population of CD25<sup>+/high</sup> cells with low expression of CD4 (CD4<sup>dim</sup>CD25<sup>+/high</sup>; fig 1d). This population, forming a discrete cloud of cells in a CD4/CD25 plot (fig 1d), comprised fewer Foxp3<sup>+</sup>, CD127<sup>low</sup> and HLA-DR<sup>+</sup> cells, and it was considered less appropriate than the CD4<sup>dim</sup>CD25<sup>high</sup> gate (fig 1d and 1c, right panels) for analysis of Tregs. Thus, taken together the CD4<sup>dim</sup>CD25<sup>high</sup> population was considered the optimal definition of Tregs, and this gating strategy was applied in all the following investigations of Treg frequency, phenotype and function

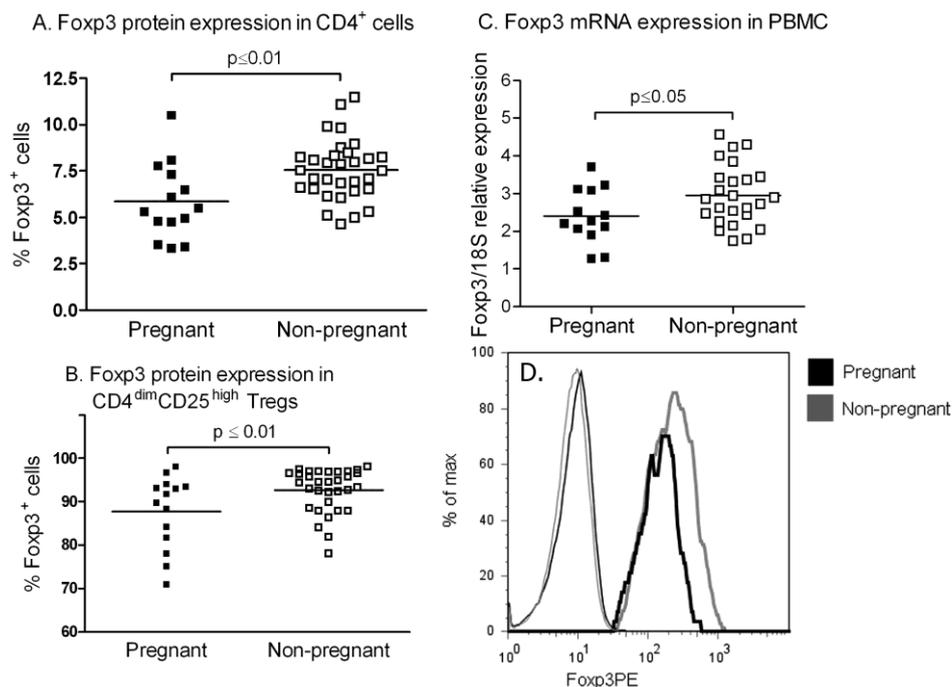
### **The CD4<sup>dim</sup>CD25<sup>high</sup> population is reduced in size during pregnancy**

As shown both by four-color and six-color flow cytometry, the percentage of circulating CD4<sup>dim</sup>CD25<sup>high</sup> cells of CD4<sup>+</sup> cells was in fact decreased in pregnancy (Four-color: 1.2±0.35% vs. 1.9±0.6%; p=0.0005; fig 1c, Six-color: 1.5±0.3% vs. 2.8±1.1%; p=0.002). In addition, TruCount analysis of absolute cell count confirmed these results showing a decrease of total CD4<sup>dim</sup>CD25<sup>high</sup> count/μL blood in pregnant compared to non-pregnant women (14±4 cells /μL vs. 21±8 cells/μL; p=0.01).

### **The CD4<sup>dim</sup>CD25<sup>high</sup> population shows a less regulatory and a more activated phenotype**

Alongside with the reduction of the CD4<sup>dim</sup>CD25<sup>high</sup> compartment in pregnancy, the CD4<sup>dim</sup>CD25<sup>high</sup> Tregs, as well as the entire CD4<sup>+</sup> population, contained a lower proportion of cells expressing Foxp3 during pregnancy (Fig 2a-2b). Similar results were obtained in the CD4<sup>+</sup>CD25<sup>+</sup> population as well as in the 0.5% CD4<sup>+</sup>CD25<sup>highest</sup> population (p=0.006 and

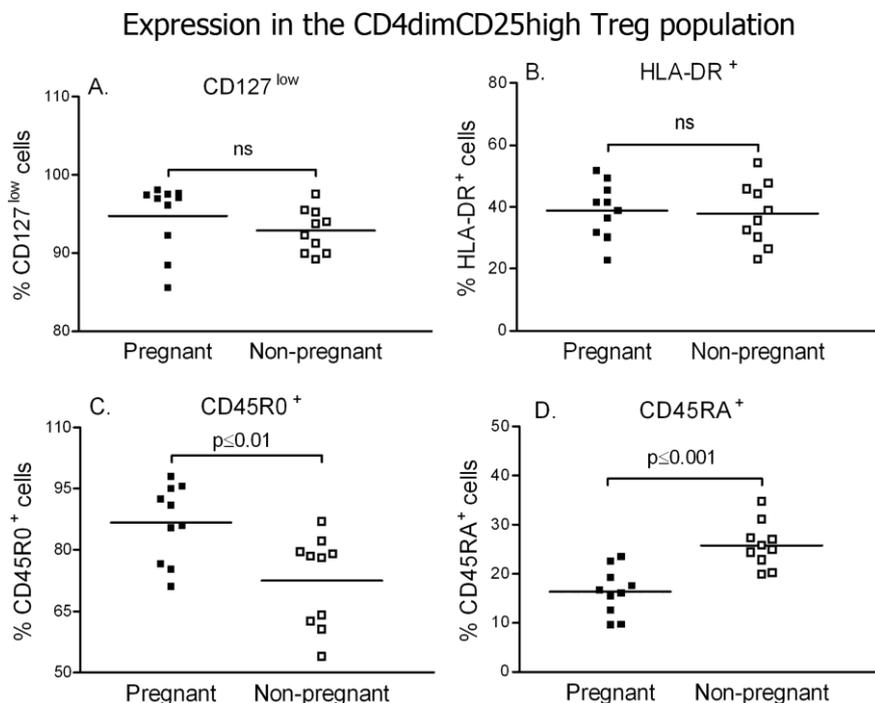
$p \leq 0.0001$ , respectively; data not shown). Also, the Foxp3 expression intensity, expressed as geometric mean fluorescence intensity (gMFI), was significantly reduced in the  $CD4^+$  as well as in the  $CD4^{dim}CD25^{high}$  population when comparing pregnant and non-pregnant women ( $p=0.003$  and  $p=0.018$ , respectively; fig 2d). In accordance with this, PBMC isolated from pregnant women showed a tendency towards lower Foxp3 mRNA expression than PBMC from non-pregnant women (relative values:  $2.4 \pm 0.7$  vs.  $2.9 \pm 0.8$ ;  $p=0.05$ ) (fig 2c).



**Figure 2.** Expression of intracellular Foxp3 protein and mRNA in PBMC from pregnant and non-pregnant women. **A.** Expression of Foxp3 protein in  $CD4^+$  cells (pregnant  $n=14$ , non-pregnant  $n=32$ ). **B.** Expression of Foxp3 protein in  $CD4^{dim}CD25^{high}$  (pregnant  $n=14$ , non-pregnant  $n=32$ ). **C.** Expression of Foxp3 mRNA in total PBMC (pregnant  $n=13$ , non-pregnant  $n=25$ ). **D.** Foxp3 fluorescence intensity (pregnant  $n=14$ , non-pregnant  $n=33$ ) where peaks to the left in the figure are Foxp3<sup>-</sup> lymphocytes (isotype controls) and peaks to the right are Foxp3<sup>+</sup> $CD4^{dim}CD25^{high}$ . This histogram overlay was constructed using the FlowJo software (Tree Star Inc., Ashland, USA) and the y-axis scale (% of max) is used to normalise for the number of events in the samples displayed. All bars represent means.

No changes in CTLA-4 expression were seen within the  $CD4^{dim}CD25^{high}$  population (data not shown). As CD27, which has been suggested a marker for Tregs in inflamed tissues (30), was highly expressed throughout the entire  $CD4^+$  population, showing no specificity for Foxp3<sup>+</sup> or  $CD4^{dim}CD25^{high}$  cells, this marker was excluded from further analysis (data not shown).

Further characterization, by six-color flow cytometry, showed that the Treg markers CD127<sup>low</sup> (fig 3a) and HLA-DR<sup>+</sup> (fig 3b) in the CD4<sup>dim</sup>CD25<sup>high</sup> Treg population was similarly expressed in pregnant and non-pregnant women. However, the frequency of HLA-DR<sup>+</sup> cells was significantly lower in pregnant women in the entire CD4<sup>+</sup> population (mean 2.6±0.9 vs. 4.1±1.3 %; p=0.007) and in the 0.5% CD4<sup>+</sup>CD25<sup>highest</sup> population (mean 53.1±9.8 vs. 67.8±8.6 %; p=0.002). When looking at the activation markers CD45R0 (effector/memory) and CD45RA (naïve), pregnant women showed an activated Treg phenotype with increased frequency of CD45R0<sup>+</sup> and decreased frequency of CD45RA<sup>+</sup> cells within the CD4<sup>dim</sup>CD25<sup>high</sup> population (fig 3c-d). No changes in the very early activation marker CD69 could be seen between pregnant and non-pregnant women and the expression of this marker was generally very low throughout the entire CD4<sup>+</sup> population (around 1%; data not shown).



**Figure 3.** Expression of A. CD127, B. HLA-DR, C. CD45R0 and D. CD45RA in CD4<sup>dim</sup>CD25<sup>high</sup> cells from pregnant (n=10) and non-pregnant (n=10) women. All bars represent means.

**The pregnancy-related changes in CD4<sup>dim</sup>CD25<sup>high</sup> Treg frequency and phenotype can be *in vitro*-induced by 17 $\beta$ -estradiol and progesterone**

PBMC from non-pregnant women were treated with 10  $\mu$ M 17 $\beta$ -estradiol and/or 200  $\mu$ M progesterone which significantly lowered the frequency of CD4<sup>dim</sup>CD25<sup>high</sup> cells and Foxp3<sup>+</sup> cells compared to untreated cells (fig 4a). This reduction of the CD4<sup>dim</sup>CD25<sup>high</sup> population, most pronounced for progesterone treatment, was observed in both anti-CD3 stimulated and unstimulated (data not shown) cultures. However, in contrast to results from pregnant women, neither progesterone nor 17 $\beta$ -estradiol induced an expansion of the classical CD4<sup>+</sup>CD25<sup>high</sup> nor the CD4<sup>high</sup>CD25<sup>high</sup> population (data not shown). Within the CD4<sup>dim</sup>CD25<sup>high</sup> population, 17 $\beta$ -estradiol and progesterone, alone or in combination, lowered the frequency of Foxp3<sup>+</sup>, CTLA-4<sup>+</sup> and HLA-DR<sup>+</sup> cells (fig 4b), whereas the expression of CD127 remained unchanged (data not shown). Again, these changes were prominently induced by progesterone and also seen in non-anti-CD3 stimulated cultures (data not shown). Further, the effects of hormone treatments were similar in the secretory and proliferative phases of the menstrual cycle. The lower concentrations of hormones (10 nM and 100 nM for 17 $\beta$ -estradiol and 200 nM and 2  $\mu$ M for progesterone) caused changes that followed the effects of the highest concentrations (10  $\mu$ M 17 $\beta$ -estradiol and 200  $\mu$ M progesterone) but were much less pronounced (all changes were not statistically significant, data not shown). Thus, only data from the highest concentrations are shown (fig 4).

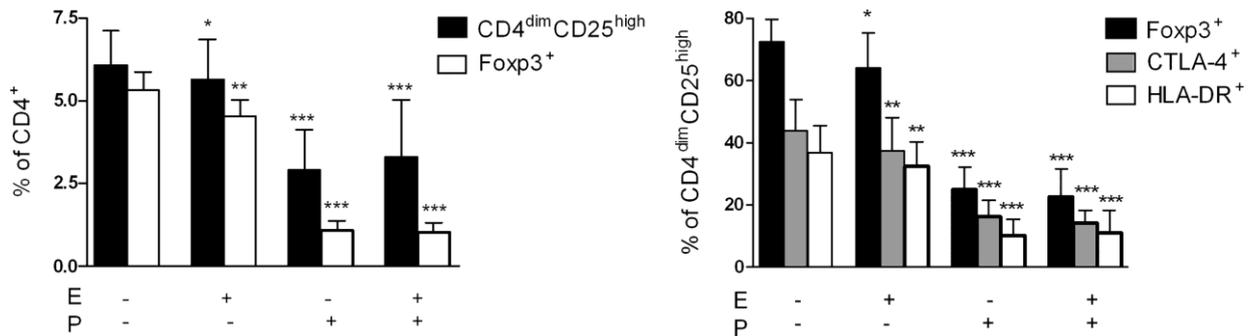


Figure 4. PBMC from non-pregnant women ( $n=7$ ) were stimulated with  $10 \mu\text{M}$   $17\beta$ -estradiol (E) and  $200 \mu\text{M}$  progesterone (P) in the presence of plate-bound  $0.005 \mu\text{g/mL}$  anti-CD3 antibody for 3 days. A. Proportion of  $\text{CD4}^{\text{dim}}\text{CD25}^{\text{high}}$  and  $\text{Foxp3}^+$  cells in the  $\text{CD4}^+$  population. B. Proportion of  $\text{Foxp3}^+$ ,  $\text{CTLA-4}^+$  and  $\text{HLA-DR}^+$  cells within the  $\text{CD4}^{\text{dim}}\text{CD25}^{\text{high}}$  population.

Bars represent means and standard deviations (SD). Significance-markers (\*) indicate differences between hormone stimulated and unstimulated cells. \*  $p \leq 0.05$ , \*\*  $p \leq 0.01$ , \*\*\*  $p \leq 0.001$ .

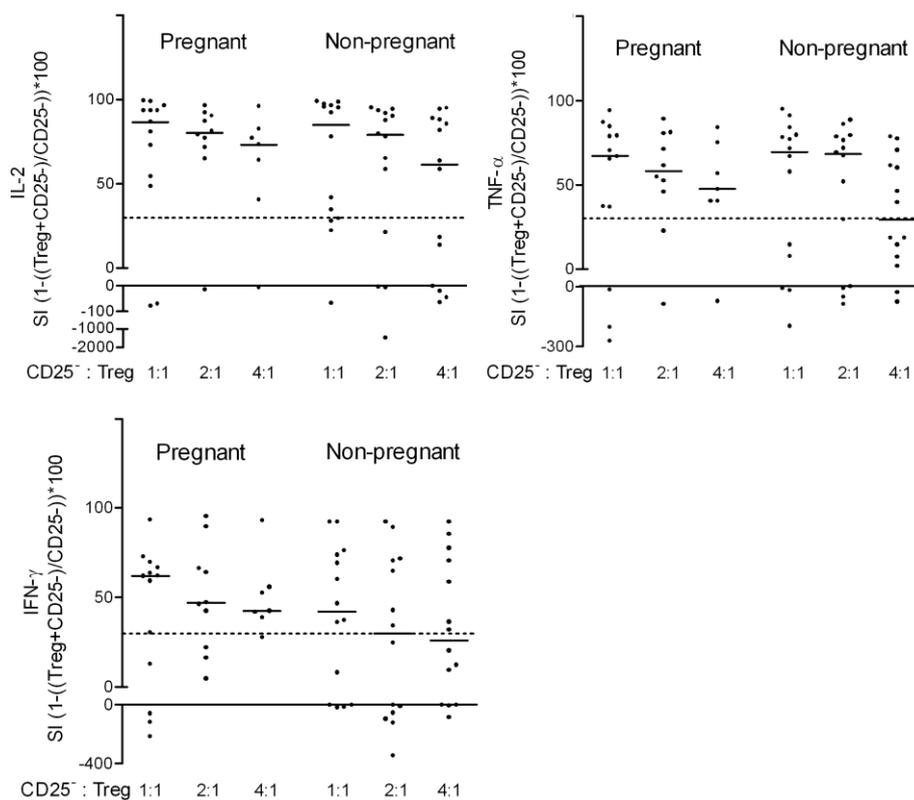
### **$\text{CD4}^{\text{dim}}\text{CD25}^{\text{high}}$ Tregs suppress secretion of IL-2, TNF- $\alpha$ and IFN- $\gamma$ but not IL-4 and IL-10 whereas $\text{CD4}^{\text{high}}\text{CD25}^{\text{high}}$ cells are completely non-suppressive**

The functional effects of  $\text{CD4}^{\text{dim}}\text{CD25}^{\text{high}}$  Tregs from pregnant and non-pregnant women were evaluated in a system with plate-bound  $\alpha$ -CD28 ( $5 \mu\text{g/mL}$ ) and  $\alpha$ -CD3 antibodies at two different concentrations (suboptimal:  $1 \mu\text{g/mL}$ , optimal:  $5 \mu\text{g/mL}$ ). Both concentrations generated results according to the same pattern, but as the optimal  $\alpha$ -CD3 concentrations gave higher and more reliable cytokine responses, without abrogation of Treg suppressive function, data from this stimulation is shown.

$\text{CD4}^{\text{dim}}\text{CD25}^{\text{high}}$  Tregs from both pregnant and non-pregnant women suppressed the secretion of IL-2, TNF- $\alpha$  and IFN- $\gamma$  from  $\text{CD4}^+\text{CD25}^-$  cells (fig 5). Although the suppression appeared to be more pronounced with increasing numbers of  $\text{CD4}^{\text{dim}}\text{CD25}^{\text{high}}$  Tregs, no statistical differences were observed between the different Treg doses. Confirming their role as suppressors,  $\text{CD4}^{\text{dim}}\text{CD25}^{\text{high}}$  Tregs alone secreted less IL-2, TNF- $\alpha$  and IFN- $\gamma$  compared to  $\text{CD4}^+\text{CD25}^-$  alone or when co-cultured with  $\text{CD4}^+\text{CD25}^-$  cells (fig 6a-c). Despite the reduced

expression of Foxp3 in  $CD4^{dim}CD25^{high}$  Tregs in pregnancy, the ability of the Tregs to suppress IL-2, TNF- $\alpha$  and IFN- $\gamma$  secretion was similar in pregnant and non-pregnant women, both when analyzed as cytokine concentrations and as suppressive indexes (SI).

Tregs from pregnant and non-pregnant women did not suppress the secretion of IL-4 or IL-10 (fig 6d-6e). In fact, supernatants from  $CD4^{+}CD25^{-}$ /Treg co-cultures and Tregs alone contained significantly more IL-4 than supernatants from  $CD25^{-}$  cells cultured alone (fig 6d).  $CD4^{+}CD25^{-}$ /Treg co-cultures from pregnant women tended to produce more IL-4 than co-cultures from non-pregnant women ( $p=0.06$ ). As for IL-4,  $CD4^{dim}CD25^{high}$  Tregs from pregnant, but not from non-pregnant women tended to secrete more IL-10 than  $CD4^{+}CD25^{-}$  cells alone (fig 6 e).



*Figure 5. The suppressive capacity of FACSaria sorted  $CD4^{dim}CD25^{high}$  Tregs in co-culture with autologous  $CD4^{+}CD25^{-}$  responder cells from pregnant ( $n=7-13$ ) and non-pregnant women ( $n=14$ ) expressed as suppressive index ( $1 - (\text{secretion in co-culture} / \text{secretion from } CD4^{+}CD25^{-} \text{ cells alone}) \times 100$ ).*

*Solid lines indicate median values and dotted lines indicate the estimated intra-assay variation (30%).*

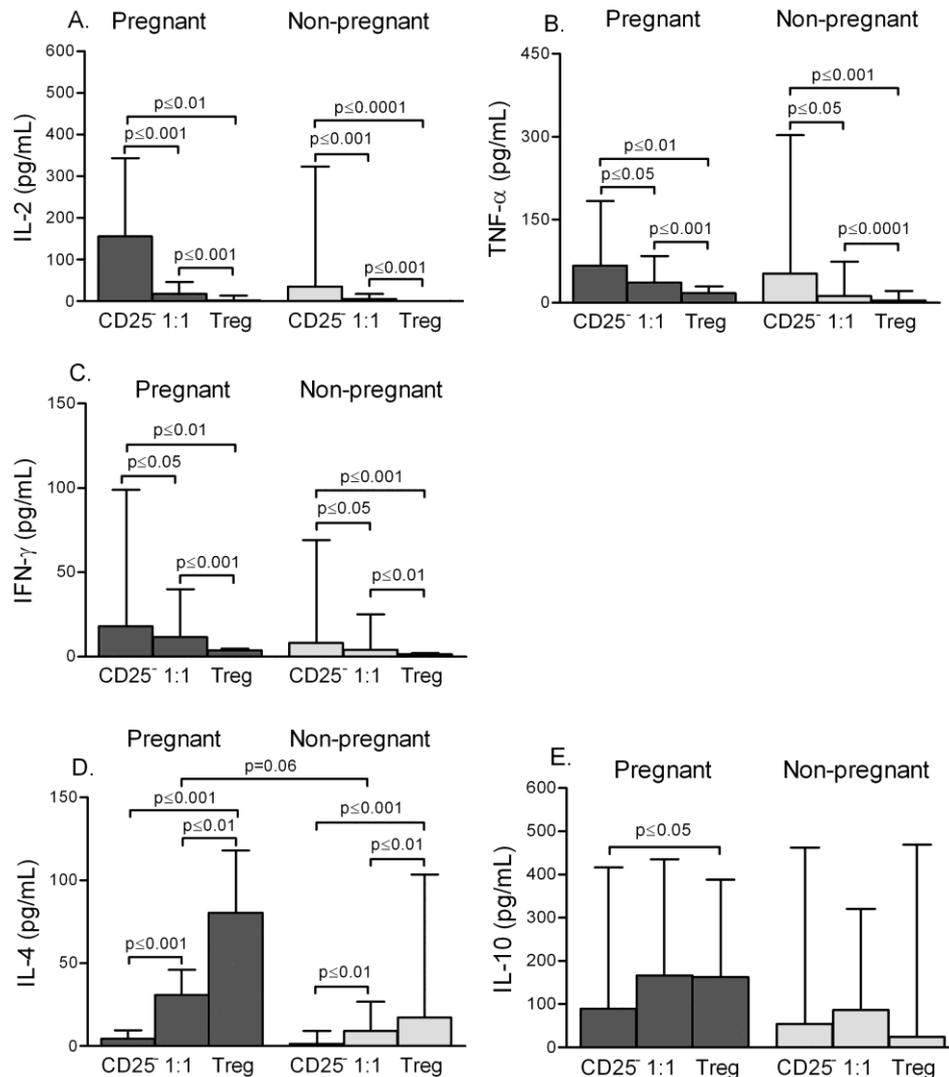
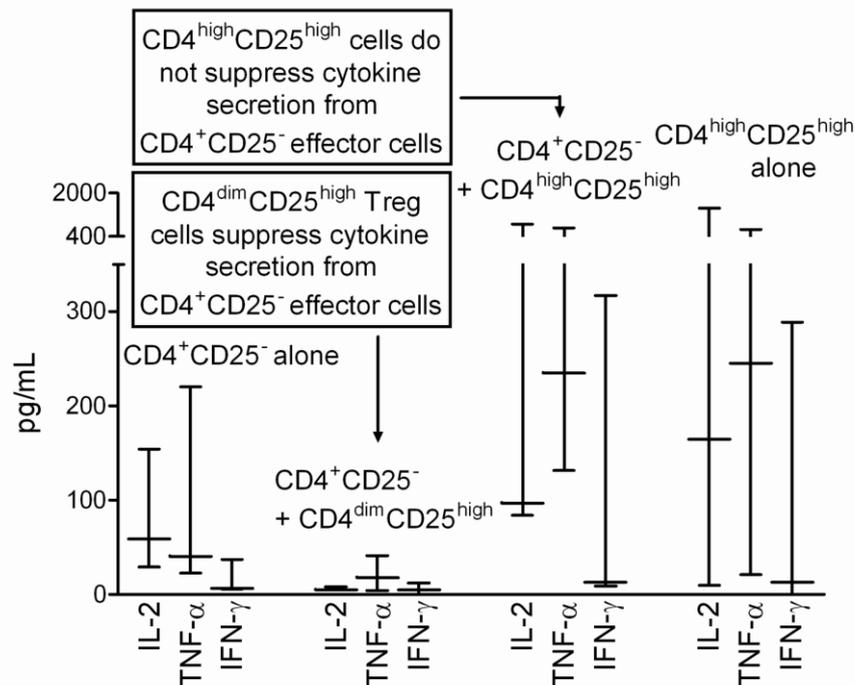


Figure 6. The secretion of A. IL-2, B. TNF- $\alpha$ , C. IFN- $\gamma$ , D. IL-4 and E. IL-10 from CD4<sup>+</sup>CD25<sup>-</sup> cells alone, CD4<sup>dim</sup>CD25<sup>high</sup> Tregs alone, or in 1:1 combination. Dark grey filled bars show data from pregnant women (n=13) and light grey filled bars show data from non-pregnant women (n=14). Bars represent medians and the 75<sup>th</sup> percentile.

CD4<sup>high</sup>CD25<sup>high</sup> cells (fig 1b) from pregnant women, defined as having higher CD4 expression than CD4<sup>dim</sup>CD25<sup>high</sup> Tregs (fig 1c), yet falling into the classical CD4<sup>+</sup>CD25<sup>high</sup> gate (fig 1a), did not suppress the secretion of IL-2, TNF- $\alpha$  and IFN- $\gamma$  (fig 7). Rather, CD4<sup>high</sup>CD25<sup>high</sup> cells secreted high levels of IL-2, TNF- $\alpha$  and IFN- $\gamma$  (fig 7), as well as IL-4 (median 1147 pg/mL; min 87 pg/mL, max 2413 pg/mL) and IL-10 (median 2294 pg/mL; min 15 pg/mL, max 2574 pg/mL), hence contributing to an increase in the bulk amount of these

cytokines in co-culture with  $CD4^+CD25^-$  cells. Thus, this confirmed the phenotypic data and further strengthened the notion that  $CD4^{high}CD25^{high}$  cells are not suppressive Tregs.



*Figure 7. Lack of suppressive capacity in the pregnancy-associated population  $CD4^{high}CD25^{high}$  ( $n=3$ , i.e. data is shown as min, max and median values). The secretion of IL-2, TNF- $\alpha$  and IFN- $\gamma$  from  $CD4^+CD25^-$  cells alone (far left) is suppressed by Tregs ( $CD4^{dim}CD25^{high}$  cells; left) but not by  $CD4^{high}CD25^{high}$  cells (right) in 2:1 (responder : suppressor) combination. The secretion from  $CD4^{high}CD25^{high}$  cells (25 000 cells,  $n=1$ ; or 6 250 cells,  $n=2$ ) alone are shown to the far right in the figure.*

## Discussion

To evaluate the role for Tregs, in any condition, the defining of an accurate flow cytometric gate for analysis and sorting of these cells is a key prerequisite. In this study we thoroughly assessed the Treg gating strategy and thereby defined a distinct  $CD4^{dim}CD25^{high}$  population with high prevalence of Foxp3<sup>+</sup>, CD127<sup>low</sup> and HLA-DR<sup>+</sup> cells. By applying this gating strategy we found, in contrast to most previous studies characterizing Tregs only by CD4 and CD25 expression, that circulating  $CD4^{dim}CD25^{high}$  Tregs were reduced in second trimester

normal pregnancy. Further, CD4<sup>dim</sup>CD25<sup>high</sup> Tregs from pregnant women also showed an altered phenotype, with reduced levels of Foxp3. These changes could be replicated *in vitro* by progesterone and, albeit to a lesser extent, also by 17 $\beta$ -estradiol, by treating PBMC from non-pregnant women with these hormones. Importantly, despite lowered Foxp3 expression, Tregs from pregnant women maintained their suppressive function and clearly suppressed IL-2, TNF- $\alpha$  and IFN- $\gamma$  secretion from CD4<sup>+</sup>CD25<sup>-</sup> cells. Further, Tregs from both pregnant and non-pregnant women secreted considerable amounts of IL-4 and IL-10, a finding that was most apparent in pregnant women and could help explain their maintained suppressive function.

There are several suggestions as to the cause of the Treg modifications seen during pregnancy, the most prevailing being presence of fetal antigens and altered hormonal milieu. Our results draw attention to progesterone as a potent modulator of Tregs. We showed that progesterone, and to a lesser extent 17 $\beta$ -estradiol, were able to induce the here reported alterations in circulating Tregs in pregnancy, i.e. a reduction in frequency and an altered phenotype. We used hormone concentrations that are higher but comparable to those found physiologically at the fetal maternal interface during pregnancy (31, 32). It should be noted that reliable information about local progesterone and 17 $\beta$ -estradiol concentrations is scarce. Importantly, hormone concentrations corresponding to serum levels (33, 34) caused slight but statistically not consistently significant changes that followed the very pronounced effects seen at higher concentrations. Expression of estrogen and progesterone receptors have been identified in various immune cells (35, 36) but to our knowledge, only receptors for estrogen have been confirmed on Tregs (24).

Our observations regarding 17 $\beta$ -estradiol are in contrast to the general consensus that in the murine system, Tregs are potentiated by 17 $\beta$ -estradiol (16, 37, 38). Interestingly, under

certain inflammatory conditions, human pregnancy levels of  $17\beta$ -estradiol have been shown to enhance the expression of NF- $\kappa$ B (39), one of the targets for Foxp3 suppression (40), in fact pointing towards a counteracting effect of  $17\beta$ -estradiol on Tregs, in line with our results. However, it was recently shown that  $17\beta$ -estradiol increased the proliferation and function of human Tregs (24). The discrepancies between the studies may be explained by differences in purity of the sorted Tregs, formulation of the  $17\beta$ -estradiol used and also strength of the TCR-stimulation in the *in vitro* assay. We used highly pure flow cytometry sorted Tregs, positive for Foxp3 expression, and water-soluble  $17\beta$ -estradiol thereby avoiding the background cell activation caused by ethanol-soluble  $17\beta$ -estradiol. Further, since pregnancy is a situation of alloantigen “awareness”, our *in vitro* system with low grade of TCR stimulation seems more physiologically representative of pregnancy. Recently, Tregs and levels of  $17\beta$ -estradiol, but not progesterone, were shown to correlate during the menstrual cycle (41). However, as both  $17\beta$ -estradiol and progesterone increase dramatically during pregnancy (33) and the effects of  $17\beta$ -estradiol seem to be concentration-dependent (42), the influence of these hormones might well be different during pregnancy. Our study suggests that even though both progesterone and  $17\beta$ -estradiol have important and well documented immune modulating effects on pregnancy (43, 44), these do not seem to be mediated via the promotion of Tregs.

We found an increased  $CD4^{\text{high}}CD25^{\text{high}}$  population in pregnant women (fig 1b), probably being responsible for previous estimations of an increased circulating Treg population in pregnancy. Importantly, we showed that this population was non-suppressive and since it did not expand in response to neither progesterone nor  $17\beta$ -estradiol, the expansion of this population must be caused by other pregnancy-related changes, possibly by the presence of fetal alloantigens. Previous studies in humans have shown  $CD4^+CD25^{\text{high}}$  Treg frequencies (expressed as percentage of  $CD4^+$ ) ranging from 8 to 17.5% in pregnant and 4.4-10% in non-

pregnant women (19, 21-23). With support of more refined Treg markers, such as Foxp3, CD127<sup>low</sup> and HLA-DR, we argue that CD4<sup>+</sup>CD25<sup>high</sup> numbers of that magnitude (> mean 1.2% in pregnant women) will likely include a population of Foxp3<sup>-</sup>, CD127<sup>+</sup> and HLA-DR<sup>-</sup> cells (CD4<sup>high</sup>CD25<sup>high</sup> cells). Functional and phenotypical studies revealed that the CD4<sup>high</sup>CD25<sup>high</sup> cells were not suppressive but rather activated cells secreting high levels of all cytokines investigated. Hence, including CD4<sup>high</sup>CD25<sup>high</sup> cells in a Treg gate would lead to misinterpretations, not only of Treg frequency and phenotype, but also of functional characteristics. Taken together, we suggest that previous findings on expanded circulating Tregs in pregnancy need re-evaluation. Furthermore, we stress the importance of using a strict Treg gate, preferably based on co-expression of several Treg markers, to obtain reliable data not only in pregnancy, but also in other immune challenging conditions such as autoimmune diseases and transplantations.

We report that Tregs in pregnant women display reduced Foxp3 expression, a finding recently acknowledged also by Tilburgs and colleagues (45). Further, Tregs found in pregnant women seem more activated (increased CD45RO<sup>+</sup> and reduced CD45RA<sup>+</sup> frequencies) compared with non-pregnant women. Despite their altered phenotype, circulating Tregs from second trimester pregnant women were able to potently suppress the secretion of the proinflammatory cytokines TNF- $\alpha$  and IFN- $\gamma$  as well as IL-2 to the same extent as non-pregnant women. However, in pregnant women, we did observe a tendency towards an increased ability of Tregs, alone or in co-culture, to secrete IL-4 and IL-10. Interestingly, pregnant women displayed a lower proportion of cells expressing HLA-DR in the 0.5% CD4<sup>+</sup>CD25<sup>highest</sup> and the CD4<sup>+</sup> cell population compared to controls, which is in line with a very recent report (45). In the context of Treg immune regulation, HLA-DR on CD4<sup>+</sup>CD25<sup>high</sup> cells can distinguish between two distinct Treg populations (11), where HLA-DR<sup>-</sup> Tregs secrete cytokines like IL-

4 and IL-10 and suppress in a late contact-dependent manner *in vitro*. Our data suggest that in pregnancy, the Treg population, which holds the 0.5% CD4<sup>+</sup>CD25<sup>highest</sup> population, comprise a higher proportion of HLA-DR<sup>-</sup> Treg cells with the potential of secreting cytokines such as IL-4 and also IL-10. This could be the explanation for the maintained suppressive function, despite reduced Foxp3 expression, of pregnancy-associated Tregs. Interestingly, this is in accordance with the general view of pregnancy as a T<sub>H</sub>2-like phenomenon (46-49).

Pregnant women showed maintained Treg suppressive function, despite a reduced frequency of Foxp3<sup>+</sup> cells within the CD4<sup>dim</sup>CD25<sup>high</sup> population, as well as in the total PBMC population, both at the mRNA- and protein-level. This is somewhat of a paradox since Foxp3 is believed to correlate with suppressive function. However, recent data suggest that Foxp3 is not as specific for regulatory T cells as first thought, since it was shown that transient Foxp3 expression may actually be induced by *in vitro* stimulation of non-regulatory T cells (5-7). Importantly, stable, but not transient, expression of Foxp3 leads to down-regulation of the IL-7 receptor CD127, a target gene for Foxp3 which correlates with suppressive function (9, 10). We did not observe a decreased proportion of CD4<sup>dim</sup>CD25<sup>high</sup> cells showing the CD127<sup>low</sup> phenotype, indicating that during pregnancy, Foxp3 is stable and still capable of suppressing CD127 expression. Further, using mouse strains that exhibit Tregs with reduced or no Foxp3 expression has led to the understanding that Foxp3 expression is not an on-off switch but that Foxp3 works along a continuum, inducing increasing grades of suppressive properties (50, 51). Interestingly, it was shown that Foxp3<sup>low</sup> cells develop into T<sub>H</sub>2 like effector cells secreting IL-4 (51). It is tempting to draw parallels between these Foxp3 deficient Tregs and Tregs from pregnant women since Tregs, alone or in coculture, tended to secrete more IL-4 and IL-10 in pregnant as compared to non-pregnant women. Although these findings need

further confirmation, this could be a mechanism by which maintained systemic tolerance is achieved, without the expansion of the Treg population.

In this study, we analyzed circulating Tregs, whereas the situation at the fetal-maternal interface may be different. The observed reduction in circulating Tregs could in fact be a consequence of a local recruitment to the decidua/placenta where Tregs seem enriched (20) and ought to have a more obvious role in protecting the fetus against detrimental immune reactions. During the writing of this manuscript, Tilburgs and colleagues reported that fetus-specific Treg cells could only be found at the fetal-maternal interface (45), whereas we previously found indications of fetus-specific Tregs in the circulation (18). Thus, it is tempting to speculate that during pregnancy, non-fetus specific Tregs are down-regulated systemically to ensure optimal maternal defence against infections.

The pregnant women included in this study all displayed healthy pregnancies upon inclusion. However, one woman delivered prematurely in gestational week 27. Data obtained from this woman did not diverge from the overall data pattern except for one point: In contrast to all the other pregnant women, Tregs from this woman did not secrete higher levels of IL-4 than did CD25<sup>-</sup> T effector cells. We find this intriguing and with reservation to the fact that this is an isolated observation from a single patient, this may indicate a role for Treg-associated IL-4 production in healthy pregnancy. However, this finding has to be confirmed.

In conclusion, we show that circulating Tregs, defined as CD4<sup>dim</sup>CD25<sup>high</sup>Foxp3<sup>+</sup>, are reduced in second trimester human pregnancy. However, Tregs from pregnant women are still potently suppressing IL-2, TNF- $\alpha$  and IFN- $\gamma$  secretion in co-culture with CD4<sup>+</sup>CD25<sup>-</sup> responder cells, while efficiently producing IL-4 and IL-10. In an *in vitro* system resembling

the pregnancy hormonal milieu,  $17\beta$ -estradiol and, in particular, progesterone induced a reduction of  $CD4^{\dim}CD25^{\text{high}}Foxp3^+$  cells in PBMC from non-pregnant women. Our findings support the view of hormones, especially progesterone, as critical regulators of the  $Foxp3^+$  Treg population in pregnancy. Further, the current study suggests that systemic tolerance during pregnancy is not facilitated by an increased Treg activity and that early data on this topic may need re-evaluation.

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### **Disclosures**

The authors declare no conflict of interest or financial interests.

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## Footnotes

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<sup>3</sup> Non-standard abbreviations

TCM; T cell culture medium, Tregs; Regulatory T cell, Foxp3; Forkhead box P3, gMFI; geometric mean fluorescence intensity.