Adaptation and Modulation of Memory and Regulatory T Cells in Pregnancy
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A Different Immune Phenotype in Decidual Tissue from Multigravid Women Compared to Primigravid Women

Manuscript in preparation

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ABSTRACT

Women with a previous uncomplicated pregnancy have lower complication risks of immune-associated pregnancy disorders in a subsequent pregnancy. This could indicate a different maternal immune response in multigravid women compared to primigravid women. In a previous study, we showed persistent higher memory T cell proportions with higher CD69 expression after healthy pregnancies. However, no studies have reported on memory T cells in multigravid and primigravid women. Therefore, we compared memory T cell subsets and their CD69 expression in multigravid women and multigravid women. Lymphocytes were isolated from term decidua parietalis and decidua basalis tissue from healthy primigravid women (n=12) and multigravid women (n=12). Using flow cytometry, central- (CCR7+), effector- (CCR7-), tissue resident- (CD103+), regulatory- (FOXP3+) memory (CD45RO+), and regulatory- (FOXP3+) T cell populations and their CD69+ proportions were analyzed in the CD4+ and CD8+ cell compartments. All T cell subsets analyzed in the decidua parietalis had significantly higher CD69+ proportions in multigravid women compared to primigravid women. In the decidua basalis, trends towards higher CD4+ Treg and CD4+ Treg memory cell proportions were observed in multigravid women compared to primigravid women. To analyze whether a different immune phenotype is already present in early decidual tissue, decidual tissue from uncomplicated ongoing pregnancies between 9 and 12 weeks of gestation was investigated using qRT-PCR to analyze mRNA expression. Higher FOXP3 mRNA expression and a trend towards higher HLA-DR mRNA expression was found in multigravid women compared to primigravid women. In conclusion, this study shows that decidual tissue of multigravid women has a different immune phenotype compared to decidual tissue of primigravid women in early pregnancy and at term which could suggest altered immune regulation.

INTRODUCTION

Women with a previous successful pregnancy have lower risks of pregnancy complications with suspected immunological etiology such as fetal growth restriction and pre-eclampsia in their next pregnancy. These complications of pregnancy are associated with aberrant immune responses, which may not be able to completely tolerate the semi-allogeneic fetus. The mechanisms underlying fetal-maternal tolerance are incompletely understood, but adaptations of a variety of immune cells are necessary during pregnancy to suppress an immune rejection response. For instance in the decidua, tolerance towards fetal cells is important since there is direct contact between fetal trophoblast cells and maternal immune cells. In the decidua parietalis,
which is the uterine lining located around the fetal membranes, there is interaction of immune cells with chorionic trophoblasts\textsuperscript{8}, while in the decidua basalis, the maternal part of the placenta, there is interaction of the immune cells with extravillous cytotrophoblasts\textsuperscript{9}. The decidua parietalis and the decidua basalis each contain a different immune cell repertoire\textsuperscript{10–13}.

Memory T cells in decidual tissue are thought to be important for fetal-maternal tolerance\textsuperscript{10,12,14,15}. Memory T cells are capable of memorizing antigens to elicit a more enhanced response upon reactivation by the cognate antigen\textsuperscript{16}. A variety of memory T cell subsets can be recognized based on their function and migration potential; i.e. central-memory (CM), effector-memory (EM), tissue-resident memory (TRM), and regulatory T (Treg) memory cells\textsuperscript{14}. We have previously shown that memory T cell populations in peripheral blood of women are persistently altered by healthy pregnancy, with higher proportions CD4\textsuperscript{+} memory T cells and higher CD69\textsuperscript{+} proportions of CD4\textsuperscript{+} memory T cells during pregnancy and postpartum compared to nulligravid women\textsuperscript{17}. Studies in mice have shown that CD4\textsuperscript{+} Treg memory cells are imprinted with the fetal-paternal antigen during pregnancy, remain latent postpartum and reaccumulate in a subsequent pregnancy, decreasing fetal resorption rates\textsuperscript{18}. Although various human studies in pregnancy investigated the Treg cell population by using transcription factor forkhead box P3 (FOXP3)\textsuperscript{19–21}, the memory Treg cell population is hardly studied in human pregnancy\textsuperscript{22}.

The higher CD69\textsuperscript{+} proportions of CD4\textsuperscript{+} memory T cells may suggest activation of these cells, since various studies have associated CD69 expression with T cell activation\textsuperscript{23,24}. These previous data may be in line with studies showing increased expression of activation markers on memory T cells in uncomplicated pregnancies such as human leukocyte antigen-DR (HLA-DR)\textsuperscript{25,26}, CD38\textsuperscript{25}, lymphocyte activation gene-3 (LAG-3)\textsuperscript{15}, and inhibitory costimulatory molecule T cell immunoglobulin and mucin domain-3 (TIM-3)\textsuperscript{27}. However, more recent insights into CD69 expression have shown that it may have specific functions on selected T cells subsets. It was found that CD69 enhances immune regulatory abilities of Treg cells\textsuperscript{28}, suppresses Th1 and Th17 cell differentiation\textsuperscript{29,30}, is necessary for adequate memory T cell formation\textsuperscript{31,32}, and that CD69 plays a role in retention of TRM cells in tissues\textsuperscript{33}.

The reasons for the persistent changes of memory T cell populations and its CD69 expression during and after pregnancy remain unknown. However, as multigravid women have lower risks of immune associated pregnancy complications compared to primigravid women, we propose that these changes may have beneficial effects in
a subsequent pregnancy. Therefore, this study aims to analyze memory T cells and their CD69 expression in decidual tissue of primigravid and multigravid women. This was investigated by isolating T cells from the decidua parietalis and basalis tissue at term to perform flow cytometric analysis on these cells. QRT-PCR analysis was done to detect mRNA expression of CD69 and T cell activation and regulation markers in decidua basalis tissue from early pregnancy of primigravid and multigravid women.

Table 1. Characteristics term decidual tissue

<table>
<thead>
<tr>
<th></th>
<th>Primigravid women (n = 12)</th>
<th>Multigravid women (n = 12)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal age (years)</td>
<td>29.0 (25.5-32.5)</td>
<td>34.5 (33.0-36.0)</td>
<td>0.00</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>22.1 (19.1-25.1)</td>
<td>21.7 (18.5-24.9)</td>
<td>1.00</td>
</tr>
<tr>
<td>Gravidity</td>
<td>1</td>
<td>3.0 (2.13-3.88)</td>
<td>0.00</td>
</tr>
<tr>
<td>Parity</td>
<td>0</td>
<td>1.0 (0.5-1.5)</td>
<td>0.00</td>
</tr>
<tr>
<td>Gestational age (weeks)</td>
<td>40.5 (39.65-41.35)</td>
<td>40.2 (38.65-41.75)</td>
<td>0.77</td>
</tr>
<tr>
<td>Mode of delivery (% vaginal)</td>
<td>100</td>
<td>91.7</td>
<td>0.31</td>
</tr>
<tr>
<td>Birth weight (grams)</td>
<td>3360 (3077-3643)</td>
<td>3475 (3015-3935)</td>
<td>0.33</td>
</tr>
<tr>
<td>Fetal sex (% female)</td>
<td>58.3</td>
<td>50.0</td>
<td>0.68</td>
</tr>
<tr>
<td>Time in PBS (minutes)</td>
<td>1035 (711-1359)</td>
<td>1017 (713-1321)</td>
<td>0.73</td>
</tr>
</tbody>
</table>

Continuous variables are displayed as median (interquartile range), percentages are shown as % of total. Statistical analysis by Mann-Whitney U test or Chi-square test.

**METHODS**

**Term decidual tissue samples**

Term decidual tissue was collected from 12 primigravid women who reported a first pregnancy, and 12 multigravid women who reported at least 1 uncomplicated previous pregnancy. All women were healthy, had uncomplicated pregnancies, had a BMI <33kg/m², did not smoke, did not drink alcohol, and did not use drugs or immune modulatory medication. If known, multigravid women who were pregnant from a new partner were excluded.
For acquiring term decidual tissue samples, the code of conduct for responsible use following the guideline from the Federation of Medical Scientific Associations was followed\textsuperscript{34}. The study, the consent procedure, and the objection procedure against use of medical waste for research purposes were approved by the Medical Ethics Review Board of the University Medical Center Groningen (UMCG) (protocol number METc2018/516).

**Isolation of lymphocytes from term decidual tissue**

Within one hour after delivery, the placenta and membranes were stored at 4°C in phosphate buffered saline (PBS) for a maximum of 24 hours according to a previously published protocol\textsuperscript{35}. To separate the decidua parietalis from the fetal membranes, the amniotic membrane was removed by gentle traction, after which the decidua parietalis was scraped of the chorionic membrane using a cell scraper (Corning incorporated, USA). All visible blood vessels were manually removed from the decidua parietalis to prevent contamination with red blood cells. To isolate the decidua basalis, the villi were cut from the placenta and as much villi as possible were removed. Both tissues were mechanically and enzymatically digested using a GentleMACS dissociator (Miltenyi, Germany) in a 1:2 dilution of StemPro Accutase (LifeTechnologies, USA). Thereafter, a Percoll gradient (Sigma-Aldrich, USA) was used to isolate lymphocytes from the interface between densities of 1.053 g/ml – 1.080 g/ml. Cells were counted using a Beckman coulter counter (Beckman Coulter, USA). After counting, cells were resuspended in decomplemented fetal calf serum (dFCS) with 20% dimethylsulfoxide (DMSO) and were brought to -80°C in cryotubes using a MrFrosty (Thermo Fisher Scientific, USA). After 24 hours, the cryotubes with the cells were transferred to liquid nitrogen and stored until further analysis.

**Flow cytometry**

Lymphocytes from term decidual tissue were thawed in a 37°C water bath and resuspended in Roswell Park Memorial Institute 1680 (RPMI) medium (Lonza, USA). For viability staining, 0.001% Fixable Viability Stain 620 (BD Biosciences, USA) in PBS was added to cell aliquots of 10⁶ cells per well in a 96-wells plate (Corning, USA). Cells were incubated at room temperature in the dark for 15 minutes. To prevent nonspecific binding, cell aliquots were incubated with 50 µl 1% Fc Block (BD Biosciences, USA) and 10% mouse serum (Sanquin, The Netherlands) at room temperature for 10 minutes. Extracellular antibodies APC-H7 anti-CD4 (SK3, BD Biosciences, USA), PE-Cy7 anti-CD8 (RPA-T8, BD Biosciences, USA), BV510 anti-CD45RO
(UCHL-1, BD Biosciences, USA), PE anti-CCR7 (150503, BD Biosciences, USA), APC anti-CD69 (FN50, BD Biosciences, USA), and BV421 anti-CD103 (BER-ACT8, BD Biosciences, USA) were added to the cell aliquots and incubated on ice for 30 minutes. To allow for intracellular staining, Fixation/Permeabilization buffer (BD Biosciences, USA) was used according to manufacturer’s instructions. Then, intracellular antibody AF488 anti-FOXP3 (2326A/E7, BD Biosciences, USA) was added to the cell aliquots and cells incubated on ice for 30 minutes. Thereafter, cells were resuspended in PBS and analyzed on a FACSVerse flow cytometer (BD Biosciences, USA) using BD FACS Suite software (BD Biosciences, USA). For data analysis, FlowJo V10 software (LLC, USA) was used. Figure 1 shows the gating strategy used for flow cytometric analysis.

First, single cells were selected (Figure 1A). In a forward sideward scatter of single cells, a lymphocyte gate was set (Figure 1B). Within the lymphocyte population, live lymphocytes were selected by excluding cells positive for the viability stain (Figure 1C). Within the live lymphocyte population, CD4+ and CD8+ cells were identified (Figure 1D). Within the CD4+ cell compartment, Treg (FOXP3+) cells (Figure 1E) were gated. Within both the CD4+ and the CD8+ cell populations, EM (CD45RO+CCR7-) and CM (CD45RO+CCR7+) cells were selected (Figure 1F, 1I). Within the CD4+ cell compartment, Treg memory cells (FOXP3+CD45RO+) were selected (Figure 1G). Within the CD8+ cell compartment no FOXP3+ cells were observed in the decidual

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**Table 2. Characteristics early pregnancy decidual tissue**

<table>
<thead>
<tr>
<th></th>
<th>Primigravid women (n = 13)</th>
<th>Multigravid women (n = 13)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>At CVS</strong></td>
<td></td>
<td></td>
<td>---------</td>
</tr>
<tr>
<td>Maternal age (years)</td>
<td>37.0 (34.5-39.5)</td>
<td>37.0 (35.5-38.5)</td>
<td>0.71</td>
</tr>
<tr>
<td>Gestational age (days)</td>
<td>10.8 (10.2-11.5)</td>
<td>11.1 (11.3-11.3)</td>
<td>0.61</td>
</tr>
<tr>
<td>Gravidity</td>
<td>1</td>
<td>2.0 (1.5-2.5)</td>
<td>0.00</td>
</tr>
<tr>
<td>Parity</td>
<td>0</td>
<td>1.0 (0.5-1.5)</td>
<td>0.00</td>
</tr>
<tr>
<td><strong>At delivery</strong></td>
<td></td>
<td></td>
<td>---------</td>
</tr>
<tr>
<td>Gestational age (weeks)</td>
<td>41.0 (40.0-42.0)</td>
<td>41.0 (40.0-42.0)</td>
<td>0.96</td>
</tr>
<tr>
<td>Birth weight (grams)</td>
<td>3500 (3121-3879)</td>
<td>3620 (3287.5-3952.5)</td>
<td>0.37</td>
</tr>
<tr>
<td>Fetal sex (% female)</td>
<td>23.1</td>
<td>30.8</td>
<td>0.66</td>
</tr>
</tbody>
</table>

Continuous variables are displayed as median (interquartile range), percentages are shown as % of total. Statistical analysis by Mann-Whitney U test or Chi-square test.
layers. Within the CD8+ cell compartment, TRM cells (CD103+CD45RO+) were selected (Figure 1J). Identification of CD4+ TRM cells with CD103 is under debate and is therefore not included in this study. Within all T cell subsets, CD69+ proportions were identified using a positive gate for CD69 (Figure 1H, 1K). Due to the low number of lymphocytes that could be isolated from the term decidual tissue samples, no fluorescence minus one staining could be performed.

**Figure 1.** Gating strategy of flow cytometric analysis of decidual T cells at term. Representative dot plots showing the gating strategy for single cells (A), lymphocytes (B), live lymphocytes (C), CD4+ and CD8+ cells (D), CD4+ Treg (FOXP3+) cells (E), CD4+ EM (CD45RO-CCR7-) and CM (CD45RO-CCR7+) cells (F), CD4+ Treg memory (CD45RO-FOXP3+) cells (G), CD69+ cells of CD4+ memory cells (H), CD8+ EM (CD45RO-CCR7-) and CM (CD45RO-CCR7+) cells (I), CD8+ tissue resident memory (CD45RO-CD103+) cells (J), and CD69+ cells of CD8+ memory cells (K) in the decidua parietalis.

Early pregnancy decidua basalis samples

Decidual tissue from waste material of chorionic villous samples was used for qRT-PCR analysis. Chorionic villous samples (CVS) were taken from women between 9 and 12 weeks of gestation (confirmed by ultrasound). CVS was indicated for maternal age or serum screening related risk of Down syndrome. Samples were taken using a biopsy catheter (Cook, K-CMA-5000). After collection of the tissue, villi were mechanically removed from the decidua basalis under the microscope to prevent trophoblast contamination. Villous tissue was used for screening purposes, while decidual tissue was considered waste material and could be used for qPCR analysis. Decidual tissue was stored until further analysis at -20°C following the protocol of Huisman et al. Karyotype analysis showed no abnormalities in any of the samples. A total of 26 samples (13 primigravid and 13 multigravid) were selected from a tissue database and matched for gestational age, maternal age, and fetal
sex. None of the women smoked, used medication, underwent assisted reproductive techniques, and all women had successful pregnancies confirmed by a questionnaire completed postpartum. For acquiring first trimester decidual tissue samples, the code of conduct for responsible use following the guideline from the Federation of Medical Scientific Associations was followed.

![Figure 2](image-url)

**Figure 2.** T cell populations in the decidua parietalis and the decidua basalis from primigravid (PG) women and multigravid (MG) women at term. Proportions of CD4+ cells of the lymphocyte population (A), T regulatory (Treg) cells (FOXP3+) of the CD4+ cell population (B), CD8+ cells of the lymphocyte population (C), CD69+ proportions of the CD4+ cell population (D), the CD4+ Treg cell population (E), and the CD8+ cell population (F). Symbols represent individual values per decidua with data as median with interquartile range. Analysis by Mann-Whitney U test to compare PG and MG women in the decidua parietalis and in the decidua basalis. *p < 0.05, **p < 0.01.

**qRT-PCR**

Early pregnancy decidua basalis samples were thawed at room temperature and Qiazol lysis reagent (Qiagen, USA) was added. A Tissuelyser (Qiagen, USA) was used to disrupt and homogenize the samples (2 minutes, 50 Hertz). RNA was isolated using
RNeasy plus mini kit (Qiagen, USA) following manufacturer’s instructions. RNA quantification was performed using a NanoDrop Spectrophotometer (ND-1000, Nanodrop Technologies, USA). cDNA was reverse transcribed using Superscript-II Reverse Transcriptase kit (Invitrogen, USA). mRNA expression of markers previously associated with memory T cell activation in uncomplicated pregnancies; CD69 (Hs00934033_m1)\(^{17}\), LAG-3 (Hs00158563_m1)\(^{15}\), HLA-DR (Hs00219575_m1)\(^{25}\), CD38 (Hs01120071_m1)\(^{25}\), inhibitory costimulatory molecule TIM-3 (Hs00958618_m1)\(^{27}\), immune regulatory associated transcription factor FOXP3 (Hs01085834_m1), and housekeeping gene hypoxanthine-guanine phosphoribosyltransferase (HPRT) (Hs02800695_m1) were analyzed using Taqman On-Demand-Gene-Expression Assays (Thermo Fisher Scientific, USA). HPRT mRNA expression was used as a housekeeping gene as the CT values were normally distributed, did not differ statistically between the groups, and had a lower overall standard deviation compared to beta-actin (ACTB) mRNA expression\(^{41}\). PCR reactions were performed on a VIIA7-Real-Time PCR System (Thermo Fisher, USA) in triplicates with 15 ng cDNA per reaction. Data were normalized to gene expression of housekeeping gene HPRT using \(2^{-\Delta C_T}\).

**Statistical analysis**

All data were tested for normality using the Kolmogorov-Smirnov test. Outliers were excluded using the ROUT method\(^{42}\). A Mann-Whitney U test was performed to compare data from primigravid women and multigravid women. A Chi-square test was used to compare categorical parameters. Linear regression analysis was performed to detect possible associations of T cell proportions with maternal age. GraphPad Prism version 7 for Microsoft Windows (GraphPad software Inc., USA; Microsoft, USA) and IBM SPSS version 23.0 software for Microsoft Windows (SPSS Inc, USA) were used for data analysis. Differences were considered statistically significant when \(p < 0.05\). \(p\) values < 0.10 were considered a statistical trend.

**RESULTS**

**Baseline characteristics term decidual tissue samples**

Term decidual tissue of 12 primigravid women and 12 multigravid women were analyzed within 24 hours after birth (Table 1). Primigravid women, did not report a previous pregnancy, whereas multigravid women had a median of 1 pregnancy before the current pregnancy. Primigravid women were younger than multigravid women with a median difference of 5.5 years (\(p < 0.01\)). No differences were found
between the primigravid and the multigravid group for maternal BMI, gestational age, mode of delivery, birth weight, fetal sex, and storage time of the samples in PBS.

Higher decidual CD8+ cell proportions and higher CD69+ T cell proportions in multigravid women compared to primigravid women at term

To analyze differences in maternal T cell populations between primigravid women and multigravid women, flow cytometric analysis was performed on lymphocytes isolated from decidua parietalis and decidua basalis tissue of uncomplicated pregnancies at term.

In the decidua parietalis, no differences were found between multigravid women and primigravid women for the general CD4+ cell proportions and the CD4+ Treg cell

Figure 3. T cell populations in the decidua parietalis and the decidua basalis from primigravid (PG) women and multigravid (MG) women at term. Proportions of CD4+ memory cells (CD45RO+) of the CD4+ cell population (A), CD4+ central memory (CM) cells (CCR7+CD45RO+) of the CD4+ cell population (B), CD4+ effector memory (EM) cells (CCR7-CD45RO+) of the CD4+ memory cell population (C), CD4+ T regulatory (Treg) memory cells (FOXP3+CD45RO+) of the CD4+ cell population (D), CD69+ proportions of the CD4+ memory cell population (E), the CD4+ CM cell population (F), the CD4+ EM cell population (G), and the CD4+ Treg cell population (H). Symbols represent individual values per decidua with data as median with interquartile range. Analysis by Mann-Whitney U test to compare PG and MG women in the decidua parietalis and in the decidua basalis. *p < 0.05, **p < 0.01.
proportions (Figure 2A, 2B), but significantly higher general CD8+ cell proportions were found in multigravid women compared to primigravid women ($p < 0.01$) (Figure 2C). Significantly higher CD69+ proportions of the general CD4+ cell population ($p < 0.05$), the CD4+ Treg cell population ($p < 0.01$) and the general CD8+ cell population ($p < 0.05$) were observed in the decidua parietalis of multigravid women compared to primigravid women (Figure 2D, 2E, 2F).

In the decidua basalis, the general CD4+ cell proportions did not differ between multigravid women and primigravid women (Figure 2A). A trend towards higher proportions of CD4+ Treg cells ($p = 0.07$) was found in the decidua basalis of multigravid women compared to primigravid women (Figure 2B). Higher general CD8+ cell proportions were found in the decidua basalis of multigravid women compared to primigravid women ($p < 0.01$) (Figure 2C). The CD69+ proportions of the general CD4+ cell population in the decidua basalis were comparable between the groups (Figure 2D), but CD4+ Treg cells had significantly higher CD69+ proportions in multigravid women compared to primigravid women in the decidua basalis ($p < 0.01$) (Figure 2E). A trend towards higher CD69+ proportions was observed in the general CD8+ cell population in the decidua basalis of multigravid women compared to primigravid women ($p = 0.09$) (Figure 2F).

Higher CD69+ proportions of CD4+ memory cells in the decidua parietalis of multigravid women compared to primigravid women at term

We analyzed different subsets of CD4+ memory T cells and their CD69+ proportions in the decidua basalis and decidua parietalis at term. In the decidua parietalis, the general CD4+ memory cell, CD4+ CM cell, CD4+ EM cell, and CD4+ Treg cell proportions did not differ between multigravid women and primigravid women (Figure 3A, 3B, 3C, 3D). The CD69+ proportions of the general CD4+ memory cell ($p < 0.05$), CD4+CM cell ($p < 0.05$), CD4+ EM cell ($p < 0.01$), and CD4+ Treg memory cell population ($p < 0.01$) were significantly higher in the decidua parietalis of multigravid women compared to primigravid women (Figure 3E, 3F, 3G, 3H).

In the decidua basalis, proportions of general CD4+ memory cells, CD4+ CM cells and CD4+ EM cells were comparable in primigravid women and multigravid women (Figure 3A, 3B, 3C). A trend towards higher CD4+ Treg memory cell proportions was observed in the decidua basalis of multigravid women compared to primigravid women ($p = 0.07$) (Figure 3D). The CD69+ proportions of the general CD4+ memory
cell, CD4+ CM, and CD4+ EM cell population in the decidua basalis did not differ between multigravid women and primigravid women (Figure 3E, 3F, 3G). Significantly higher CD69+ proportions of the CD4+ Treg memory cell population were found in the decidua basalis of multigravid women compared to primigravid women (p < 0.05) (Figure 3H).

Lower CD8+ CM cell proportions and higher CD69+ proportions of CD8+ memory cells in the decidua parietalis of multigravid women compared to primigravid women at term

CD8+ memory T cell subsets were analyzed in the decidua basalis and decidua parietalis of primigravid women and multigravid women at term. In the decidua parietalis, general CD8+ memory cell proportions and CD8+ TRM cell proportions did not differ between the primigravid and multigravid group (Figure 4A, 4D). Significantly lower CD8+ CM cell proportions were found in the decidua parietalis of multigravid women compared to primigravid women (p < 0.05) (Figure 4B). A trend towards higher CD8+ EM cell proportions was observed in multigravid women compared to primigravid women in the decidua parietalis (p = 0.09) (Figure 4C). All CD8+ memory cell subsets had significantly higher CD69+ proportions in multigravid women compared to primigravid women in the decidua parietalis (Figure 4E, 4F, 4G, 4H).

In the decidua basalis, the general CD8+ memory cell, CD8+ EM cell and CD8+ TRM cell proportions were similar between multigravid women and primigravid women (Figure 4A, 4C, 4D). A trend towards lower CD8+ CM cell proportions was found in the decidua basalis of multigravid women compared to primigravid women (p = 0.06) (Figure 4B). The CD69+ proportions of all CD8+ cell subsets analyzed were similar between multigravid women and primigravid women in the decidua basalis (Figure 4E, 4F, 4G, 4H).

Baseline characteristics early decidua basalis samples

Multigravid women had a median of 1 pregnancy before the current pregnancy, whereas primigravid women did not report a previous pregnancy. No differences were observed between the primigravid and multigravid group for maternal age at CVS, gestational age at CVS, gestational age at delivery, fetal birth weight, and fetal sex. The BMI of the women in this cohort is unfortunately unknown.
Higher FOXP3 mRNA expression in early pregnancy decidual tissue from multigravid women compared to primigravid women

In first trimester decidual tissue from primigravid women and multigravid women mRNA expression of CD69, CD38, LAG-3, and TIM-3 were similar (Figure 5A, 5B, 5D, 5E). A trend towards higher mRNA expression of HLA-DR was observed in multigravid women compared to primigravid women (p = 0.06). Significantly higher FOXP3 mRNA expression was observed in multigravid women compared to primigravid women in early pregnancy decidual tissue (p < 0.01) (Figure 5F).

DISCUSSION

In this study, we compared memory T cell subsets and their CD69 expression in term decidual tissue from primigravid women with multigravid women. In the
decidua parietalis at term, we observed higher CD8+ cell proportions, lower CD8+ CM cell proportions and higher CD69+ proportions of all CD4+ and CD8+ T cell (memory) subsets analyzed in this study of multigravid women compared to primigravid women. In the decidua basalis at term, we observed higher CD8+ cell proportions, higher CD69+ proportions of CD4+ Treg and CD4+ Treg memory cells, and trends towards higher CD4+ Treg and CD4+ Treg memory cell proportions in multigravid women compared to primigravid women. In addition, in first trimester decidua basalis tissue, we observed higher FOXP3 mRNA expression and a trend towards higher HLA-DR mRNA expression in multigravid women compared to primigravid women. Although we have shown differences in memory T cell subpopulations after pregnancy in peripheral blood, to our knowledge, no other study reported on the differences between memory T cell populations in primigravid versus multigravid women. Several studies did provide evidence for a CD8+ memory cell subset with HY-specificity following a pregnancy with a male fetus43–45. It was suggested that these persisting CD8+ memory T cells might play a role in a following pregnancy, however human studies investigating this are lacking. Our human study may be in line with a mouse study by Barton et al.46. Barton et al. also did not find expansion of the CD8+ memory T cell population in subsequent pregnancies in mice. They found lower proliferative capacity and lower cytokine producing capacity of this specific T cell population46. Apparently, the CD8+ memory T cell population does not

![Image of Figure 5](image)

**Figure 5.** mRNA expression of genes encoding activation markers and immune regulatory transcription factor Forkhead box P3 (FOXP3) in decidual tissue of primigravid (PG) women and multigravid (MG) women between 9 and 12 weeks of gestation. mRNA expression of CD69 (A), CD38 (B), human leukocyte antigen-DR (HLA-DR) (C), lymphocyte activation gene-3 (LAG-3) (D), T cell immunoglobulin and mucin domain-3 (TIM-3) (E), and FOXP3 (F). Symbols represent individual values per decidua with data as median with interquartile range. Analysis by Mann-Whitney U test to compare PG and MG women. **p < 0.01.
expand in a subsequent pregnancy, but its functionality might be different. The latter has to be studied further in human subjects with functional tests comparing memory T cell populations from primigravid and multigravid women.

We found higher CD69\(^+\) proportions in all memory T cell subsets in the decidua parietalis as well as in the CD4\(^+\) Treg memory and CD4\(^+\) Treg cell population in the decidua basalis of multigravid women compared to primigravid women. Although CD69 is classically seen as an activation marker, it has also been shown to be important for several effector functions of T cells, including immune regulatory functions\(^{29,30,47}\). Higher CD69\(^+\) proportions in multigravid women compared to primigravid women could therefore suggest a more immune regulatory response in multigravid women. The presence of such an immune regulatory response is in line with the trend towards higher CD4\(^+\) Treg memory and CD4\(^+\) Treg cells in the decidua basalis of multigravid women compared to primigravid women. Similar results were found in a mouse study from Rowe et al. who reported a CD4\(^+\) Treg memory cell population with fetal antigen specificity which re-accumulated in a subsequent pregnancy lowering fetal resorption rates\(^{18}\). An increased immune regulatory environment in the second pregnancy could already be present early in pregnancy, since we observed higher FOXP3 mRNA expression in early decidual tissue from multigravid women compared to primigravid women.

To analyse whether the different regulatory immune phenotype between primigravid and multigravid women is already present in decidual tissue in early pregnancy, we investigated decidual tissue from first trimester chorionic villous samples from uncomplicated primigravid and multigravid ongoing pregnancies. Due to the small amount of tissue available, flow cytometric analysis was unfortunately not possible, and qRT-PCR could only be performed for a limited number of genes. We observed a trend towards higher mRNA expression of HLA-DR and significantly higher FOXP3 mRNA expression in decidual tissue of multigravid women compared to primigravid women. The trend towards higher HLA-DR mRNA expression may suggest activation of memory T cells. However, various other activated immune cells also express HLA-DR, such as activated T cells and activated antigen presenting cells, for instance monocytes\(^{48}\). Therefore, this trend towards HLA-DR upregulation in multigravid women compared with primigravid women suggests an increased general activation of immune cells in these women. Moreover, upregulation of HLA-DR on CD4\(^+\) and CD8\(^+\) regulatory T cells is associated with increased immune suppressive capabilities\(^{49-51}\). Interestingly, we also found higher FOXP3 mRNA expression in multigravid women compared to primigravid women which may suggest increased immune regulation in these women.
Further studies are necessary to identify whether increased immune activation, immune regulation or a combination of both is present in the early decidua of multigravid women compared to primigravid women and which cells are involved.

In this study, the multigravid and primigravid groups in both cohorts were carefully matched, but the median maternal age of the multigravid group was 5.5 years higher compared to the primigravid group in the cohort of women who donated term decidual tissue. Linear regression analysis did not show an association between maternal age and T cell proportions and their activated frequencies. Therefore, we do not consider this as a confounding variable. Since the samples from first trimester and term decidua are processed differently and are from different cohorts of women, comparison of the parameters between the two cohorts cannot be performed.

In conclusion, we show a different immune phenotype in decidual tissue of primigravid women compared to multigravid women. We found significantly higher proportions of CD69 expressing memory T cells in the decidua parietalis and a trend towards higher CD4+ Treg memory cell proportions in the decidua basalis at term from multigravid women compared to primigravid women. In addition, we found higher FOXP3 mRNA and a trend towards higher HLA-DR mRNA expression in the decidua basalis in early pregnancy in multigravid women compared to primigravid women. These findings might suggest a more regulatory immune phenotype in multigravid women compared to primigravid women. Whether this is also true for the early pregnancy decidua and whether this contributes to enhanced fetal-maternal tolerance and lowering pregnancy complication risks in a subsequent pregnancy remains to be investigated in longitudinal studies with larger sample sizes and functional analyses.
REFERENCES


