Adaptation and Modulation of Memory and Regulatory T Cells in Pregnancy
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Lower activation of CD4+ memory T cells in preeclampsia compared to healthy pregnancies persists postpartum

Submitted

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ABSTRACT

Insufficient adaptations of the maternal immune system are associated with pregnancy complications such as preeclampsia and its recurrence risk. Therefore, peripheral blood samples were taken from healthy pregnant women \( (n=15) \), preeclamptic pregnant women \( (n=15) \), formerly healthy pregnant women \( (n=16) \), and formerly preeclamptic women \( (n=15) \). CD4\(^+\) and CD8\(^+\) memory cells (CD45RO\(^+\)), central-memory cells (CM, CD45RO\(^+\)CCR7\(^+\)), effector-memory cells (EM, CD45RO\(^+\)CCR7\(^-\)), and their activated (CD69\(^+\)) proportions were analyzed using flow cytometry. A magnetic Luminex assay was performed on plasma samples from all groups to analyze 16 cytokines associated with memory T cells homeostasis and T cell differentiation. Proportions of CD4\(^+\) and CD8\(^+\) memory cell populations did not differ between preeclamptic and healthy pregnant women or between formerly preeclamptic and formerly healthy pregnant women. However, activated proportions of the general CD4\(^+\) memory, CD4\(^+\) EM, and CD4\(^+\) CM population in the peripheral blood were lower during preeclampsia compared to healthy pregnancies and were also lower postpartum in formerly preeclamptic compared to formerly healthy pregnant women. This was accompanied by lower IL2 concentrations in plasma from formerly preeclamptic compared to formerly healthy pregnant women. The lower activated proportions of memory T cells after a preeclamptic pregnancy were not associated with altered memory T cell associated cytokine plasma concentrations. These findings showed lower activation of memory CD4\(^+\) T cell subsets in and after preeclampsia as compared with healthy pregnancies, which makes their implication in the preeclampsia recurrence risk likely.

INTRODUCTION

During pregnancy, adaptations of the maternal immune system are necessary to prevent immune rejection of the semi-allogeneic fetus. Insufficient adaptations of the maternal immune system are associated with pregnancy complications such as pregnancy loss, fetal growth restriction, and preeclampsia\(^1-3\). Preeclampsia occurs in 5-8% of pregnancies, and has short- and long term consequences for fetal and maternal health\(^4,5\). Long term consequences on maternal health include increased risk of hypertension, ischemic heart disease, and stroke\(^6,7\). Besides these consequences, pre-eclampsia has an average recurrence risk of 10-20%\(^8,9\). Many factors are implicated in the pathophysiology of preeclampsia, but increasing evidence shows that especially insufficient adaptations of the maternal immune system are involved\(^10-12\).
Several immune mechanisms are implicated in the pathophysiology of preeclampsia, including alterations in levels and function of regulatory T cells. Another implicated immune mechanism could be formation of immunologic memory; i.e. the ability of the immune system to memorize antigens, which is mainly regulated by memory T cells. Memory T cells are formed during an immune response and remain latent to induce rapid cell expansion and secretion of cytokines on a second encounter with the cognate antigen to ensure quick clearance. In peripheral blood, the main memory T cell subsets are effector-memory (EM) cells and central-memory (CM) cells with either a CD4+ or CD8+ background. Upon activation with the cognate antigen, CM cells induce quick expansion of the T cell population and differentiate into EM cells. The EM cell population also rapidly expands upon reactivation and secretes large amounts of pro-inflammatory cytokines such as interferon-gamma (IFN-gamma), interleukin-4 (IL4), and IL5.

Previous studies indicated that memory T cells may have a different function in reproduction, and might contribute to fetal-maternal tolerance, reducing risks of pregnancy complications. However, studies on memory T cells in preeclampsia are limited and contradictory. Two studies found higher levels of the general CD4+ memory T cell population in peripheral blood of women with preeclampsia compared to healthy pregnant women. Whether the differences in these studies were observed in the CM or the EM cell subset was not addressed. Moreover, another study found comparable levels of general CD4+ cells. Locally, at the fetal-maternal interface, impaired function of memory T cells has been shown in preeclamptic patients. Possibly, during a preeclamptic pregnancy memory T cell populations are formed differently, which could have effects on memory T cell populations postpartum.

We have previously shown that levels of CD4+ memory cells are higher and activated proportions are more frequent in women after a healthy pregnancy compared to nulligravid women, suggesting a persistent influence of pregnancy on the memory T cell population. We hypothesized that memory T cell populations differ in preeclampsia compared to healthy pregnancies and that these differences are also present postpartum. Therefore, in the present study we evaluated CM and EM cell populations in and after preeclamptic pregnancies versus healthy pregnancies.
MATERIALS AND METHODS

Patient samples

Peripheral blood samples were taken from healthy pregnant women (n=15), preeclamptic pregnant women (n=15), formerly healthy pregnant women (n=16), and formerly preeclamptic women (n=15). Raw data from peripheral blood memory T cell subsets from 12 women with uncomplicated pregnancies, and 13 formerly healthy pregnant women were used from a previously published study28. Pregnant women were between 25 and 35 weeks of gestation. Preeclampsia was defined as gestational hypertension with a diastolic blood pressure ≥90 mmHg and systolic blood pressure ≥140 mmHg, in two different measurements, accompanied with proteinuria ≥300 mg / 24 hours, maternal organ dysfunction, or uteroplacental dysfunction, according to the definition of the International Society for the Study of Hypertension29. Formerly pregnant women were at least 6 months after birth, did not breastfeed, and had no intra uterine contraceptive devices. Samples were taken on one of the first 8 days of the menstrual cycle or on one of the first 7 days of the week without the contraceptive pill. None of the women smoked, used drugs or alcohol. All women had a body mass index (BMI) ≤30, were between 20 and 40 years of age, reported pregnancies with only one partner, and had no known immune associated disorders other than preeclampsia. Apart from oral contraceptive pill use in formerly pregnant women, only the pregnant women with preeclampsia used medication (antihypertensive drugs and/or corticosteroids for fetal lung maturation). Blood samples were taken in a 10ml lithium heparin tube (BD Biosciences, USA).

This study was approved by the Medical Ethical Committee of the University Medical Center Groningen (protocol number: NL46127.042.13 and NL55954.042.15). All women gave written informed consent.

Flow cytometry

Blood samples were processed within 1 hour after blood withdrawal. Total white blood cell (WBC) count was measured on a Sysmex, PocH 100-i (Sysmex, Japan). Thereafter, the sample was centrifuged (250 x g, 10 min, 20°C) to separate plasma from blood cells. Plasma was used for Magnetic Luminex assay. The volume of plasma was replaced with PBS (Phosphate Buffered Saline), after which ammonium chloride was added, followed by 10 min incubation on ice, to lyse red blood cells. The sample was centrifuged (600 x g, 5 min, 4°C), the supernatant was removed, and the red blood cell lysis steps were repeated. Cells were washed with FACS buffer (PBS and 2% Fetal Calf Serum). Remaining cells were counted using a coulter counter.
(Beckman, USA), and 1.000.000 cells were added per well in a 96 well plate for flow cytometric staining. Cells were blocked using 20% mouse serum (Sanquin, The Netherlands) in FACS buffer to reduce non-specific binding\textsuperscript{30}. After centrifugation (600 \times g, 3 min, 4°C) and removal of supernatant, cells were incubated (30 min, 4°C) with monoclonal antibodies CD3 (APC-Efluor 780, SK7) (eBioscience, USA), CD4 (FITC, OKT4) (BioLegend, USA), CD8 (Perpc-cy5.5, RPA-T8) (BioLegend, USA), CD45RO (PeCy7, UCHL1) (BioLegend, USA), CCR7 (BV605, G043H7) (BioLegend, USA), and CD69 (PE, FN50) (BioLegend, USA). Thereafter, cells were washed with FACS buffer and fixed using FACS fix solution (BD biosciences, USA). Approximately 500.000 cells per sample were acquired for analysis using a FACSVerse flow cytometer (BD Biosciences) using BD FACS Suite\textsuperscript{TM} software (BD Biosciences). UltraComp eBeads (eBiosciences, USA) stained with a single monoclonal antibody were used for compensation settings. Isotype controls were used to control for non-specific binding and fluorescence minus one controls were used to set gates.

Data were analyzed using Flowjo V10 software (LLC, USA) (Figure 1). First, single cells were gated and lymphocytes were selected using forward and sideward scatterplots (Figure 1A, 1B). Within the lymphocyte population, CD3\textsuperscript{+} cells were selected (Figure 1C) followed by identification of CD4\textsuperscript{+} and CD8\textsuperscript{+} cells (Figure 1D). CD4\textsuperscript{+} and CD8\textsuperscript{+} cells were further subdivided into CM and EM cell populations by using CD45RO and CCR7 expression (Figure 1E, 1G). Within the different memory cell populations, CD69 expression was used to identify activated memory T cell proportions (Figure 1F, 1H).

**Magnetic Luminex Assay**

Plasma was centrifuged again (1000 \times g, 4°C) and was stored in cryotubes at -80°C until analysis. Magnetic Luminex assay (R&D systems, USA) was used to analyze concentrations of cytokines associated with memory T cell formation (IL4, IL7, IL15, and IL23\textsuperscript{15,31–34}) or cytokines associated with Th1, Th2, or Th17 differentiation (IFN-gamma, IL1-beta, IL2, IL5, IL6, IL10, IL12, IL17-alpha, IL18, IL21, and IL27\textsuperscript{35–39}). A single measurement was performed per sample according to manufacturer’s instructions. In brief, plasma was diluted in a 1:2 dilution in Calibrator Diluent RD6-52 (R&D systems, USA). Microparticle Cocktail (R&D systems, USA) was added to each individual sample and incubated on a shaker overnight (800 rpm, 4°C). Samples were washed and Biotin-Antibody Cocktail (R&D systems, USA) was added to each sample and incubated on a shaker for 1 hour (800 rpm, 20°C). Samples were washed and incubated with Streptavidin-PE (R&D systems, USA) for 30 minutes.
Samples were washed and resuspended in 100 μl Wash Buffer (R&D systems, USA), and analyzed with a Luminex 100/200 system (R&D systems, USA) with xPONENT software (R&D systems, USA). A standard curve was created on each plate with each concentration in duplo.

**Figure 1.** Flow cytometric analysis of memory T cell subsets in peripheral blood. First, single cells were gated from doublets (A). Lymphocytes were selected in a forward/sideward (FSC/SSC) scatter-plot of single cells (B). Within the lymphocyte population, CD3+ cells were gated (C). Then, CD4+ and CD8+ cells were distinguished (D). Within the CD4+ cell population, CD4+ central memory (CM) cells (CCR7+CD45RO-CD4+) (E), CD4+ effector memory (EM) cells (CCR7-CD45RO-CD4+) (E), and general CD4+ memory cells (CM+EM) (CD45RO-CD4+) (E), were identified. Within each CD4+ memory cell subset activated (CD69+) proportions were identified (F). Within the CD8+ cell population, CD8+ CM cells (CCR7+CD45RO-CD8+) (G), CD8+ EM cells (CCR7-CD45RO-CD8+) (G), general CD8+ memory cells (CM+EM) (CD45RO-CD8+) (G), were identified. Within each CD8+ memory cell subset activated (CD69+) proportions were identified (H).
Figure 2. General lymphocyte populations in and after preeclamptic and healthy pregnancies. Proportions of lymphocytes (% of white blood cells) (A), proportions of T-lymphocytes (CD3+) (% of lymphocytes) (B), proportions of CD4+ cells (C), activated (CD69+) proportions of CD4+ cells (D), proportions of CD8+ cells (E), activated (CD69+) proportions of CD8+ cells (F) in peripheral blood of healthy pregnant women, pregnant women with preeclampsia, formerly healthy pregnant women, and formerly preeclamptic women. CD4+ and CD8+ cell proportions are shown as proportion of the CD3+ cell population. Symbols represent individual values per patient with data shown as median with interquartile range. Analysis by Kruskal-Wallis and Dunn’s test for multiple comparisons; **p < 0.01, ***p < 0.001.

Statistics

Data were tested for normality using Kolmogorov-Smirnov test. As some of the data sets were nonparametric, all data are shown as median with interquartile range.
Outliers were excluded from the data using the ROUT method. A Kruskal-Wallis test with Dunn’s test for multiple comparisons was performed to compare the healthy pregnant group with the preeclamptic group, the healthy pregnant group with the formerly healthy pregnant group, the preeclamptic group with the formerly preeclamptic group, and the formerly healthy pregnant group with the formerly preeclamptic group. Categorical patient characteristics were analyzed using Fisher’s exact test. To investigate the effect of gestational age at onset of preeclampsia on activated memory cell proportions, a linear regression analysis was performed. Data were analyzed using Graphpad Prism 6.0h for mac OS X, (GraphPad Software, USA), and IBM SPSS for Windows Version 20 (IBM, USA). Differences were considered significant if \( p < 0.05 \).

RESULTS

Baseline characteristics

In table 1 an overview of the characteristics of the women enrolled in this study is shown. The maternal age, ethnicity, gestational age at blood withdrawal, day of menstrual cycle, gravidity, and fetal sex, were similar between the groups. Parity of formerly healthy pregnant women and formerly preeclamptic women was higher compared to healthy pregnant women \( (p < 0.0001) \) and preeclamptic women \( (p < 0.01) \) respectively. The median BMI in all groups was below 25 kg/m\(^2\), but the BMI of the formerly preeclamptic women was higher compared to formerly healthy pregnant women \( (p < 0.05) \). White blood cell counts were higher in the pregnant groups compared to the formerly pregnant groups, but comparisons between the preeclamptic and healthy pregnant groups, and between the formerly preeclamptic and formerly healthy pregnant groups did not show a difference. Formerly preeclamptic women delivered more through caesarean section compared to formerly healthy pregnant women \( (p < 0.01) \). All other patient characteristics did not differ between the groups.

Preeclampsia does not affect general CD4\(^+\) and CD8\(^+\) cell proportions

To investigate alterations of the general T cell subsets in and after preeclampsia, general lymphocyte, CD3\(^+\), CD4\(^+\) and CD8\(^+\) cell proportions were analyzed using flowcytometry in peripheral blood of healthy pregnant women, preeclamptic pregnant women, formerly healthy pregnant women, and formerly preeclamptic women. The lymphocyte proportion of all white blood cells was higher in formerly healthy pregnant women compared to healthy pregnant women \( (p < 0.001) \), and was also...
Table 1. Baseline characteristics

<table>
<thead>
<tr>
<th></th>
<th>Pregnant Healthy (n=15)</th>
<th>Pregnant PE (n=15)</th>
<th>Formerly Healthy Pregnant (n=16)</th>
<th>Formerly PE (n=15)</th>
</tr>
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<tbody>
<tr>
<td>Maternal age (years)</td>
<td>29.8 (28.9-33.2)</td>
<td>28.7 (25.0-32.1)</td>
<td>31.0 (28.0-35.1)</td>
<td>30.5 (26.7-33.1)</td>
</tr>
<tr>
<td>Ethnicity (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>White</td>
<td>83</td>
<td>80</td>
<td>84</td>
<td>83</td>
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<tr>
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<td>20</td>
<td>16</td>
<td>17</td>
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<tr>
<td>Gestational age at blood withdrawal (weeks)</td>
<td>30.6 (27.3-33.3)</td>
<td>30.6 (27.3-31.9)</td>
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<td>NA</td>
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<tr>
<td>Day of menstrual cycle (days)</td>
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<td>NA</td>
<td>4 (2-6)</td>
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<td>Oral contraceptive pill use (% of women)</td>
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<td>26.7</td>
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<td>Months after last delivery (months)</td>
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<td>NA</td>
<td>16.0 (11.5-23.5)</td>
<td>17.7 (13.2-32.2)</td>
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<tr>
<td>Gravidity</td>
<td>1(1-1.25)</td>
<td>1(1-2)</td>
<td>1(1-2)</td>
<td>1(1-2)</td>
</tr>
<tr>
<td>Parity</td>
<td>0 (0.0-0.25)</td>
<td>0 (0-1)</td>
<td>1 (1-2)***</td>
<td>1(1-1)†††</td>
</tr>
<tr>
<td>Body Mass Index (kg/m²)</td>
<td>23.1 (21.6-24.3)</td>
<td>25.5 (23.6-28.0)</td>
<td>21.2 (20.1-22.8)</td>
<td>24.0 (22.0-26.2)</td>
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<tr>
<td>White blood cell count (10⁹/L)</td>
<td>10.7 (8.7-11.8)</td>
<td>10.4 (8.2-15.5)</td>
<td>6.2 (5.3-7.2)***</td>
<td>5.4 (4.8-6.4)****</td>
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<tr>
<td>Gestational age at onset of preeclampsia (weeks)</td>
<td>NA</td>
<td>30.0 (26.6-31.0)</td>
<td>NA</td>
<td>34.7 (30.3-36.3)††</td>
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<tr>
<td>Preeclampsia subtype (%)</td>
<td></td>
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<td></td>
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<tr>
<td>Early-onset</td>
<td>NA</td>
<td>80</td>
<td>NA</td>
<td>40§§</td>
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<tr>
<td>Late-onset</td>
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<td>20</td>
<td>NA</td>
<td>60§§</td>
</tr>
<tr>
<td>Gestational age at delivery (weeks)</td>
<td>40.2 (39.1-41.0)</td>
<td>31.0 (28.5-33.85)****</td>
<td>40.1 (39.5-40.4)</td>
<td>35.7 (30.0-37.1)†††</td>
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<td>Corticosteroid therapy for fetal lung maturation (% of women)</td>
<td>0</td>
<td>20</td>
<td>0</td>
<td>20</td>
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<tr>
<td>Mode of delivery last pregnancy (%)</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>Vaginal</td>
<td>80</td>
<td>33.3*</td>
<td>87.5</td>
<td>40††</td>
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<tr>
<td>CS</td>
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<td>66.7*</td>
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<td>60††</td>
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<tr>
<td>Fetal sex last pregnancy (%)</td>
<td></td>
<td></td>
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<td>60</td>
<td>50</td>
<td>56</td>
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<tr>
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<td>40</td>
<td>50</td>
<td>44</td>
<td>60</td>
</tr>
</tbody>
</table>

Data as median (interquartile range); PE, preeclampsia; CS, caesarean section; Kruskal-wallis test with Dunn’s test for multiple comparisons to compare the pregnant groups, the formerly pregnant groups, the healthy pregnant with the formerly healthy pregnant group, and the preeclamptic with the formerly preeclamptic group; Mann-Whitney U-test to compare data only available in the pregnant or the postpartum group; Fisher’s exact test to compare categorical data; *Compared to healthy pregnant group; †Compared to formerly healthy pregnant group; §§Compared to preeclamptic pregnant group
higher in formerly preeclamptic women compared to preeclamptic women (p < 0.01) (Figure 2A). The lymphocyte proportion did not differ between healthy pregnant and preeclamptic women, or between formerly healthy pregnant and formerly preeclamptic women (Figure 2A). No differences were found in CD3+, CD4+, and CD8+ cell frequencies or their activated proportions between the groups (Figure 2).

Lower activated proportions of CD4+ memory cells in preeclampsia compared to healthy pregnancies persists postpartum

Memory T cell subsets were analyzed in pregnant women with and without preeclampsia, and women at least half a year postpartum after a pregnancy with or without preeclampsia. Between healthy pregnant women and preeclamptic pregnant women, during and after pregnancy, no differences were found for proportions of CD4+ memory, CD4+ CM, and CD4+ EM cells (Figure 3A, 3C, 3E). Also, no differences were observed when comparing these proportions between formerly healthy pregnant women and healthy pregnant women, or formerly preeclamptic women and preeclamptic women (Figure 3A, 3C, 3E).

Despite a lack of differences in the proportions of the CD4+ memory T cell subsets, we found clear differences in the proportions of activated memory T cell subsets. The pregnant group with preeclampsia had lower proportions of activated memory T cells (p < 0.001), CD4+ CM cells (p < 0.0001), and CD4+ EM cells (p < 0.05), compared to the healthy pregnant group (Figure 3B, 3D, 3F). In the postpartum groups, formerly preeclamptic women had lower activated proportions of CD4+ memory (p < 0.01), CD4+ CM (p < 0.001) and CD4+ EM cells (p < 0.05) compared to formerly healthy pregnant women (Figure 3B, 3D, 3F). Activated memory T cell proportions did not differ between formerly healthy pregnant women and healthy pregnant women, or between formerly preeclamptic women and preeclamptic women (Figure 3B, 3D, 3F).

CD8+ memory cell activation is not persistently affected by preeclampsia

Between healthy pregnant women and preeclamptic pregnant women, during and after pregnancy, no differences in CD8+ memory, CD8+ CM, and CD8+ EM cell proportions were observed (Figure 4A, 4C, 4E). Comparison of these proportions between healthy pregnant and formerly healthy pregnant women, or between preeclamptic and formerly preeclamptic women also did not show differences (Figure 4A, 4C, 4E).
Figure 3. CD4+ memory T cell populations in and after preeclamptic and healthy pregnancies. Proportions of general CD4+ memory cells (A), activated (CD69+) proportions of general CD4+ memory cells (B), proportions of CD4+ central memory (CM) cells (C), activated proportions of CD4+ CM cells (D), proportions of CD4+ effector memory (EM) cells (E), activated proportions of CD4+ EM cells (F) in peripheral blood of healthy pregnant women, pregnant women with preeclampsia, formerly healthy pregnant women, and formerly preeclamptic women. Memory cell proportions are shown as proportion of the CD4+ cell population. Symbols represent individual values per patient with data shown as median with interquartile range. Analysis by Kruskal-Wallis and Dunn’s test for multiple comparisons; *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
Figure 4. CD8+ lymphocyte populations in and after preeclamptic and healthy pregnancies. Proportions of general CD8+ memory cells (A), activated (CD69+) proportions of general CD8+ memory cells (B), proportions of CD8+ central memory (CM) cells (C), activated proportions of CD8+ CM cells (D), proportions of CD8+ effector memory (EM) cells (E), activated proportions of CD8+ EM cells (F) in peripheral blood of healthy pregnant women, pregnant women with preeclampsia, formerly healthy pregnant women, and formerly preeclamptic women. Memory cell proportions are shown as proportion of the CD8+ cell population. Symbols represent individual values per patient with data shown as median with interquartile range. Analysis by Kruskal-Wallis and Dunn’s test for multiple comparisons; *$p < 0.05$.

We found similar proportions of CM and EM cell subsets within the CD4+ cell compartments between preeclamptic and healthy pregnant women. Similar to our study, lower IL2 concentrations in plasma of formerly preeclamptic women compared to formerly healthy pregnant women.
Activated proportions of CD8+ memory T cells, CD8+ CM cells, and CD8+ EM cells did not differ between the healthy and preeclamptic women, or between the formerly healthy pregnant and formerly preeclamptic women (Figure 4B, 4D, 4F). Formerly preeclamptic women had significantly lower activated proportions of CD8+ EM cells compared to preeclamptic women \( (p < 0.05) \) (Figure 4F). Activated proportions of CD8+ EM cells did not differ between the formerly healthy pregnant and the healthy pregnant group (Figure 4F). No differences were observed in activated proportions of CD8+ memory cells and CD8+ CM cells between formerly preeclamptic and preeclamptic women, or between formerly healthy pregnant and healthy pregnant women (Figure 4B, 4D).

Lower IL2 plasma concentrations in formerly preeclamptic women compared to formerly healthy pregnant women

Using magnetic Luminex assay analysis, concentrations of cytokines associated with memory T cell homeostasis and Th1, Th2, or Th17 cell differentiation were analyzed in the plasma of all participating women. Lower IL2 concentrations were found in plasma of formerly preeclamptic women compared to formerly healthy pregnant women \( (p < 0.05) \) (Figure 5C). The IL2 concentrations did not differ between preeclamptic and healthy pregnant women, between formerly healthy pregnant and pregnant women, or between formerly preeclamptic and preeclamptic women. The IL6 concentration was lower in plasma of formerly preeclamptic women compared to preeclamptic women \( (p < 0.001) \) and in preeclamptic compared to formerly preeclamptic women \( (p < 0.0001) \), but concentrations did not differ between healthy and preeclamptic groups (Figure 5M). All other cytokines measured did not show any significant differences between the pregnant groups, between the postpartum groups, between the preeclamptic and formerly preeclamptic group, or between the healthy pregnant and formerly healthy pregnant group (Figure 5). Concentrations of IL4, IL5, and IL23 were below the detection level of the Luminex analysis and were therefore excluded from further analysis.

**DISCUSSION**

This study showed that activated proportions of the general CD4+ memory cell, CD4+ EM cell, and CD4+ CM cell population in the peripheral blood were lower during and after preeclampsia compared to women during and after healthy pregnancies. The lower activated memory T cell proportions after preeclampsia were accompanied...
Figure 5. Cytokine plasma concentrations in and after preeclamptic and healthy pregnancies. Interferon-gamma (IFN-gamma) (A), interleukin-beta (IL1-beta) (B), IL2 (C), IL6 (D), IL7 (E), IL8 (F), IL10 (G), IL12 (H), IL15 (I), IL17-alpha (J), IL18 (K), IL21 (L), and IL27 (M) concentrations in plasma of healthy pregnant women, pregnant women with preeclampsia, formerly healthy pregnant women, and formerly preeclamptic women. Data shown as median with interquartile range. Analysis by Kruskal-Wallis and Dunn’s test for multiple comparisons; *p < 0.05, ***p < 0.001, ****p <0.0001.
Nguyen et al. also did not find any differences for the CD4+ EM, CD8+ CM and CD8+ EM cell population between healthy and preeclamptic women, but they did report higher CD4+ CM cells in preeclamptic women compared to healthy pregnant women\(^\text{11}\). The discrepancy in the findings of the CD4+ CM cell subset in preeclampsia could be due to selection of the subtype of preeclampsia. In our study, we selected mainly early-onset preeclamptic women, while Nguyen et al. mainly included women with late-onset preeclampsia\(^\text{11}\). It has become more and more accepted that early-onset and late-onset preeclampsia are different subtypes of preeclampsia\(^\text{39}\). In our study, the median gestational age at diagnosis of preeclampsia in the formerly preeclamptic group was 34.7 weeks and the preeclamptic women had a median of 30.0 weeks and therewith our study represents more early-onset preeclampsia.

To the best of our knowledge, there are no studies on memory T cell subsets comparing postpartum women after a healthy pregnancy and postpartum women after a preeclamptic pregnancy. Some studies have reported altered maternal immune responses in formerly preeclamptic women\(^\text{42,43}\), including altered plasma cytokine levels and a different immune response after stimulation with influenza vaccination in women after preeclamptic pregnancies compared to women after a healthy pregnancy\(^\text{43–45}\). Our finding of lower IL2 plasma concentrations in formerly preeclamptic women compared to formerly healthy pregnant women is in line with these studies. However, we did not find differences in the proportions of CD4+ or CD8+ EM or CM cells in formerly preeclamptic pregnant women compared to formerly healthy pregnant women.

The main differences in our study are differences in the proportions of activated (i.e. CD69 expressing) CD4+ memory cells in both the CM and EM cell subset. We found lower proportions of these activated memory cells in preeclampsia as compared to healthy pregnancies, both during pregnancy and postpartum. The lower activated proportions we found seemed specific for the CD4+ memory T cell population, since we did not find higher proportions of CD69+ cells in the general T cell and CD8+ memory population. The exact function of CD69 is unknown, but CD69 has been found to have regulatory effects on the immune system\(^\text{46,47}\). It may inhibit inflammatory responses, since it has been shown that CD69+ suppresses the differentiation of T cells towards the Th1/Th17 cell lineage \textit{in vitro} through production of tumor growth factor beta\(^\text{47,48}\). The findings of decreased CD69 expression on various memory T cell subsets during preeclampsia as compared to healthy pregnancies may be related to the increased Th1 and Th17 responses in this condition\(^\text{49,50}\). The persistently upregulated CD69 on memory T cells in healthy pregnancies\(^\text{28}\), may be an immune
mechanism to control inflammatory responses, such as the Th1 and Th17 response, during healthy pregnancy.

An explanation for the lower CD69\(^+\) proportions in preeclampsia could be non-adequate or altered antigen presentation in preeclampsia causing altered induction of T cells and with that lower CD69 expression. In preeclampsia, altered levels and subsets of antigen presenting cells, such as dendritic cells and macrophages have been found\(^{51,52}\). Hence, antigen presentation causing differentiation and activation of T cells might be altered in preeclampsia and CD69\(^+\) expression on T cells in accordance. The lower activated proportions of memory T cells during preeclampsia also seemed to result in lower proportions of activated memory cells after preeclampsia.

An alternative explanation for the lower activated proportions of memory T cells could be an altered plasma cytokine milieu which is necessary for memory T cell formation\(^{34,53,54}\). Therefore, we measured plasma concentrations of cytokines associated with T cell differentiation, such as interferon-gamma, IL7 and IL15. We did not find differences in the levels of these cytokines between healthy pregnant and preeclamptic women during pregnancy, suggesting that the reduced activated proportions of memory T cells found in preeclampsia versus healthy pregnant women are not affected by altered levels of these plasma cytokines. We did find lower IL2 plasma concentrations in formerly preeclamptic women compared to formerly healthy pregnant women which do suggest an altered cytokine milieu postpartum after preeclampsia. It is known that IL2 is both an inducer of effector and memory responses\(^{55,56}\). Interestingly, it has also been shown that IL2 concentrations particularly affect the memory T cell subtype distribution of CD4\(^+\) cells, with higher IL2 levels leading to a decrease in CM cells\(^{57}\). Whether these higher IL2 levels are indeed linked to the reduced CD4\(^+\) memory T cell activation in these women remains to be investigated. Even though we did correct for multiple comparisons per cytokine, the results should be interpreted carefully considering the number of cytokines that was measured.

In the present study, we used strict selection criteria for preeclamptic women resulting in homogenous groups, minimizing variation, but this unfortunately also resulted in a small sample size, which could explain that we could not confirm earlier studies regarding altered cytokine levels in and after preeclampsia. 80\% of the preeclamptic women in this study had early-onset preeclampsia, whereas 40\% of the formerly preeclamptic women had early-onset preeclampsia. Interestingly, the gestational age at delivery, which reflects severity of disease, did not differ significantly between the preeclamptic and the formerly preeclamptic group. To assess whether gestational age at onset of
preeclampsia influenced the activated proportions of memory T cells, we performed linear regression analyses. No effect of gestational age at onset of preeclampsia on the activated proportions of memory T cells was found, either during pregnancy or postpartum, indicating that the gestational age of onset of preeclampsia did not affect the proportion of activated memory T cells. Due to the strict selection criteria, all formerly preeclamptic women and formerly healthy pregnant women were healthy postpartum, did not use any medication, had similar parity, had similar gravidity, and had blood withdrawn in the first 8 days of the menstrual cycle to minimize effects of sex hormone fluctuations\textsuperscript{58,59}. As the proportion of former preeclamptic women and formerly healthy pregnant women that used oral contraceptives did not differ, we believe that the use of oral contraceptives did not influence our results significantly. Although there was a statistical significant difference in BMI of formerly healthy pregnant women compared to formerly preeclamptic women (median 21.2, and 24.0 respectively), both medians were within the normal BMI range. Using linear regression analyses, we could not find an effect of BMI on the (activated) proportions of memory T cells in our study.

In conclusion, we are the first to show persistent effects of preeclampsia on the memory T cell populations. Preeclamptic women during and after preeclampsia had lower activated proportions of CD4\textsuperscript{+} memory T cells as measured by CD69 expression. These findings make the involvement of memory T cells in preeclampsia and its recurrence risk more likely. Our results encourage larger longitudinal studies, as well as functional studies in preeclampsia on memory T cells to investigate an association of memory T cells with the pathophysiology and recurrence risks of preeclampsia.
REFERENCES


Lower activation of memory T cells in preeclampsia persists postpartum