Pregnancy persistently affects memory T cell populations

Tom E.C. Kieffer, Marijke M. Faas, Sicco A. Scherjon, Jelmer R. Prins

University of Groningen, Department of Obstetrics and Gynecology, University Medical Center Groningen, PO Box 30001, 9700 RB Groningen, The Netherlands

Objectives: To study the effect of pregnancy on maternal immunity.

Methods: Central memory (CM) and effector memory (EM) CD4+ T-lymphocytes were analyzed in peripheral blood of nulligravid women prior to pregnancy and 18 months postpartum.

Results: CM and EM proportions differed significantly between the groups. During pregnancy, CM to EM proportions decreased, and CM cells were upregulated in parous women compared to nulligravid women.

Conclusions: The maternal immune system is affected by pregnancy, and this effect is more pronounced in parous women.

Keywords: Pregnancy, T cell populations, Immune system.
enables them to enter secondary lymph nodes (Förster et al., 1999; Sallusto et al., 2004). These cells display reactive behavior, they are highly sensitive for antigen stimulation and effectively stimulate dendritic cells and B cells (Sallusto et al., 2004). After activation, CD69 is expressed on the cell surface, CM cells irreversibly lose their CCR7 expression and proliferate to EM cells (Sallusto et al., 2004; Ziegler et al., 1994). Next to this, CM cells are able to renew themselves and maintain a permanent population whereas EM cell populations decrease over time (Lanzavecchia and Sallusto, 2005). EM cells do not express CCR7, therefore they are able to migrate to infectious areas (Lanzavecchia and Sallusto, 2000). Immediately after activation through antigen, EM cells express CD69 and display effector cell like behavior by producing cytokines such as interferon gamma and interleukin 4 (Lanzavecchia and Sallusto, 2000; Ziegler et al., 1994).

During childhood and adulthood most, if not all, women had multiple immune responses towards pathogens and vaccines and therefore they have a memory T cell population (Jaigirdar and MacLeod, 2015). We hypothesize that pregnancy in itself as an immune challenge leads to activation, generation and expansion of the pre-pregnancy EM and CM cell pools and that pregnancy causes persistent higher levels of EM and CM cell subsets postpartum. These persistent alterations could be of use in a subsequent pregnancy by establishing more adequate immunity during pregnancy. Although several studies have reported on memory T cells during pregnancy, persistent alterations of memory T cell populations due to pregnancy has not been shown in humans yet (Gomez-Lopez et al., 2013; Kinder et al., 2014; Piper et al., 2007; Rosenblum et al., 2015, 2011; Rowe et al., 2012; Tilburgs et al., 2010). Therefore, this study aims to evaluate the short term and the persistent effects of pregnancy on effector memory T cell and central memory T cell populations, the size of these populations and their activation status.

2. Materials and methods

2.1. Patient details

Memory T cell subsets were analyzed in peripheral blood of 14 healthy non-pregnant women that had never been pregnant before (nulligravid), 12 healthy pregnant women (primigravid) (blood withdrawal between 27 and 34 weeks of pregnancy) and 15 non-pregnant women that had been pregnant (parous) and were at least 6 months postpartum. All women were between 20 and 40 years of age, had a body mass index (BMI) between 19 and 30, did not have symptoms of illness or fever, and did not smoke. Women with fertility disorders, intra uterine contraceptive devices or immune associated diseases were excluded. All primigravid and parous women gave birth at term and had uncomplicated pregnancies.

The parous women and the nulligravid women were recruited from staff members of the University Medical Center Groningen (UMCG), and blood was collected in the first eight days of their menstrual cycle. Pregnant women were recruited from the midwife practice of Groningen and the department of Obstetrics and Gynecology of the UMCG.

This study was approved by the Medical Ethical Committee of the UMCG (protocol number: NL46127.042.13). All women gave written informed consent

2.2. Flow cytometry

A monoclonal antibody (mAb) cocktail was used to identify memory T cell subsets (Table 1). Isotype controls were used at the same concentration as the primary mAb (Table 1).

Samples were processed within one hour after blood withdrawal. Firstly, a total white blood cell count was performed using Sysmex, PoCh 100-i. Thereafter RBCs were lysed with ammonium chloride. Cells were incubated on ice for 10 min followed by centrifuging at 4 °C and RBC lysis was repeated. After the cells were washed with FACS buffer (PBS and 2% FCS), they were counted using a coulter counter (Beckman Coulter). Cells were incubated in a 96-well plate with FACS buffer and 20% mouse serum (Sanquin, Amsterdam, The Netherlands) per 1,000,000 cells to reduce nonspecific binding (Andersen et al., 2016). Cells were then centrifuged, supernatant was discarded and the mAb or isotype control cocktail was added. An unstained control was added to every row in the 96 well plate to control for possible contamination. No background signals were found in the unstained control wells. Cells were incubated on ice and were washed with FACS buffer afterwards. To preserve the staining, the cells were fixed using FACS fix solution (BD biosciences). Then, cells were resuspended in FACS buffer and analyzed with a FACSVerse™ flow cytometer (BD Biosciences) using BD FACS Suite™ software (BD Biosciences). Approximately 500,000 cells per sample were acquired for analysis.

Cells or UltraComp eBeads (eBiosciences) stained with a single mAb were used for setting compensation settings.

Data analysis was done with FlowJo v10 software (Fig. 1). T-lymphocytes were selected based on forward and side scatter plots and staining for CD3. Within the CD3+ cell population, CD4+ and CD8+ cells were distinguished. To identify memory T cells, gates were set within these populations based on CD45RO. Within the CD45RO positive population, CCR7 was used to distinguish CM and EM cells and CD69 to identify the activated cell proportion. Isotype controls and FMO controls were used to control for nonspecific characteristics of the antibodies and to set gates. Absolute cell counts of the different populations were calculated with the dual platform approach using the total white blood cell counts performed directly after blood withdrawal and the proportions calculated with FlowJo (Barnett and Reilly, 2007).

2.3. Statistics

Data were analyzed with GraphPad Prism 6.0 h for mac OS X. (GraphPad Software, CA, USA), and IBM SPSS for Windows Version 20. Statistical outliers were excluded using the ROUT method (Motulsky et al., 2006). One-way ANOVA followed by Tukey’s test for multiple comparisons was performed. To investigate the effect of parity and to control for the possible confounding effect of age, a linear regression analysis was performed. Differences were considered statistically significant if p < 0.05.

3. Results

3.1. Characteristics of donors

An overview of the characteristics of the women that participated in this study is displayed in Table 2. Parous women were on average 18 months postpartum. White blood cell counts and age differed significantly between the groups. No significant differences were found for any of the other characteristics.

Pregnancy affects the total memory T cell count and the proportion of memory T cells in the total T cell population, therefore absolute counts and proportions are displayed. The total white blood cell count is higher in primigravid women compared to both nulligravid (p < 0.0001) and parous women (p < 0.0001) (Table 2). Fig. 2A shows that the proportion of T-lymphocytes within the total white blood cell population differs between the groups. The T-lymphocyte proportion of all white blood cells is lower in primigravid women as compared with nulligravid (p < 0.0001)
Table 1
Monoclonal antibody cocktail.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Conjugate</th>
<th>Clone</th>
<th>Company</th>
<th>Marking</th>
<th>Isotype control</th>
</tr>
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<tr>
<td>CD3</td>
<td>APC-Efluor 780</td>
<td>SK7</td>
<td>eBioscience</td>
<td>T-lymphocytes</td>
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<td>OKT4</td>
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<tr>
<td>CD8</td>
<td>Perpc-Cy5.5</td>
<td>RPA-TB</td>
<td>BioLegend</td>
<td>Effector T cells</td>
<td></td>
</tr>
<tr>
<td>CD69</td>
<td>PE</td>
<td>FNS0</td>
<td>BioLegend</td>
<td>Activated T cells</td>
<td>PeCy7, Mouse IgG2a, k (BioLegend)</td>
</tr>
<tr>
<td>CD45RO</td>
<td>PeCy7</td>
<td>UCHL1</td>
<td>BioLegend</td>
<td>Memory T cells</td>
<td></td>
</tr>
<tr>
<td>CD197 (CCR7)</td>
<td>BV605</td>
<td>G043H7</td>
<td>BioLegend</td>
<td>Central memory T cells</td>
<td>BV605, Mouse IgG2a, k (BioLegend)</td>
</tr>
</tbody>
</table>

![image of FACS analysis](image)

**Fig. 1.** A representative example of a FACS data analysis. First, a gate was set around lymphocytes in the forward/sideward (FSC/SSC) scatterplot of all events (A). Lymphocytes were copied in a histogram in which CD3+ cells (T-lymphocytes) were identified (B). In a density dot plot of the CD3+ T-lymphocytes, CD4+ and CD8+ cells were distinguished (C). Then, within the CD4+ cell population (CD8+ cell population not shown), CD45RO+ cells were gated in a histogram (D); within the CD4+CD45RO+ cell population, CCR7+ (central memory) cells, CCR7- (effector memory) cells (E) and activated (CD69+) cells (F) were distinguished.

Table 2
Characteristics of donors.

<table>
<thead>
<tr>
<th></th>
<th>Nulligravid (n = 14)</th>
<th>Primigravid (n = 12)</th>
<th>Parous (n = 15)</th>
</tr>
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<tbody>
<tr>
<td>Age (years)</td>
<td>25.90 ± 0.75</td>
<td>30.03 ± 1.04</td>
<td>32.19 ± 1.24**</td>
</tr>
<tr>
<td>Gestational age (weeks)</td>
<td>3.00 ± 0.65</td>
<td>3.24 ± 0.83</td>
<td>3.69 ± 0.62</td>
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<td>Gestational age at delivery (weeks)</td>
<td>6.54 ± 0.60</td>
<td>39.97 ± 0.28</td>
<td>6.05 ± 0.31</td>
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<tr>
<td>White blood cell count (10^9/L)</td>
<td>10.51 ± 0.71***</td>
<td>10.51 ± 0.71***</td>
<td>17.67 ± 2.57</td>
</tr>
<tr>
<td>Time after delivery (months)</td>
<td>0</td>
<td>1</td>
<td>1.60 ± 0.29</td>
</tr>
<tr>
<td>Gravity</td>
<td>0</td>
<td>1</td>
<td>0.60 ± 0.29</td>
</tr>
<tr>
<td>Parity</td>
<td>0</td>
<td></td>
<td>1.60 ± 0.29</td>
</tr>
</tbody>
</table>

(mean ± SEM), analysis by one-way ANOVA followed by Tukey’s post hoc test.

* p < 0.05.
** p < 0.001.
*** p < 0.0001 compared to nulligravid women.

and parous women (p < 0.001). This makes that the T-lymphocyte count differs only slightly between the groups despite the clear difference in total white blood cell counts. Proportions of CD4+ T-lymphocytes of T-lymphocytes did not differ between the groups (Fig. 2B). However, proportions of CD8 positive cells within the T-lymphocyte population are increased in primigravid (p < 0.05) and parous women (p < 0.05) compared with nulligravid women (Fig. 2C). Absolute counts of T-lymphocytes, CD4+ cells, and CD8+ cells are shown in Fig. 2D, E, and F. A significant decrease in the T-lymphocyte count (p < 0.05) and the CD4+ cell counts (p < 0.05) was found in primigravid compared with nulligravid women (Fig. 2D and E). CD4+/CD8+ cell ratios for counts or proportions did not significantly differ between the groups (not shown).

3.2. Memory T cells

By using CD45RO and CCR7 as markers, we distinguished total memory T cells (CD45RO+), CM T cells (CD45RO+CCR7+) and EM cells (CD45RO+CCR7-) within the CD4+ and CD8+ cell populations (Fig. 1). CD69 was used as a marker to identify activated cells (Ziegler et al., 1994).

3.2.1. CD4+ memory cells

In parous women proportions of CD4+ cells that express CD45RO were significantly higher compared to nulligravid women (p < 0.001) (Fig. 3A). No difference was observed in CD45RO positive proportions of CD4+ cells in primigravid women compared
Fig. 2. T-lymphocytes distribution in nulligravid, primigravid and parous women. Proportions and absolute cell counts of T-lymphocytes (CD3+) (A and D), CD4+ cells (B and E), and CD8+ cells (C and F). Frequencies as proportions of white blood cells (WBC) (A) or T-lymphocytes (B and C). Symbols represent individual values per donor and horizontal lines indicate means ± SEM. Analysis by one-way ANOVA followed by Tukey’s post hoc test; *p < 0.05, ***p < 0.001.

Fig. 3. CD4+ memory cells in nulligravid, primigravid and parous women. Proportions and absolute cell counts of CD4+ memory (CD4+CD45RO+) cells (A and D), central memory (CM; CD4+CD45RO+CCR7+) cells (B and E) and effector memory (EM; CD4+CD45RO+CCR7−) cells (C and F). Frequencies as proportions of CD4+ cells. Symbols represent individual values per donor and horizontal lines indicate means ± SEM. Analysis by one-way ANOVA followed by Tukey’s post hoc test; *p < 0.05, **p < 0.01, ***p < 0.001.

to both nulligravid and parous women (Fig. 3A). The CM cell proportions (CD45RO+CCR7+) of CD4+ cells were substantially higher in parous women compared to both primigravid (p < 0.001) and nulligravid women (p < 0.001) (Fig. 3B). Between primigravid and nulligravid women CM cell proportions did not show any difference (Fig. 3B). The EM cell proportions (CD45RO+CCR7−) were higher in both primigravid and parous women compared to nulligravid women (p < 0.05) (Fig. 3C).

When analyzing absolute cell counts, CD4+ memory cell counts in the peripheral blood were lower in primigravid women com-
pared to both nulligravid (p < 0.01) and parous women (p < 0.05) (Fig. 3D). CD4+ CM and EM absolute cell counts showed a similar pattern, with significantly lower CM cells in primigravid women compared to both nulligravid (p < 0.01) and parous women (p < 0.05) and significantly lower EM cells in primigravid compared to nulligravid women (p < 0.05) (Fig. 3E and F).

To exclude a possible confounding effect of the age differences between the groups, a linear regression analysis was performed. This regression analysis showed that parity was significantly associated with CD4+ memory cells (p < 0.01). This association persisted after adjustment for age. Moreover, age was not significantly associated with absolute numbers or proportions of CD4+ memory cells.

### 3.2.2. CD8+ memory cells

Analysis of memory cell (CD45RO+) proportions of the CD8+ cell population revealed a significant increase in parous women compared to nulligravid women (p < 0.05) (Fig. 4A). Within the CD8+ cell population CD8+ CM (CD45RO+CCR7+) cell proportions did not differ between the groups (Fig. 4B). A significantly higher proportion of CD8+ EM (CD45RO+CCR7-) cells was found in parous women compared to nulligravid women (p < 0.05) (Fig. 4C).

Parous women had significantly higher absolute counts of both total memory T cells and EM cells compared to primigravid women (p < 0.05) (Fig. 4D and F). Similar to the proportion results no difference was seen in CM cell populations between the groups (Fig. 4E).

Linear regression analysis showed age was not significantly associated with absolute numbers or proportions of CD8+ memory cells.

### 3.2.3. Activation status of memory T cells

Analyses of the activation status by CD69 expression of cells showed that significantly higher proportions activated CD4+ cells were present in primigravid women compared to both nulligravid (p < 0.0001) and parous women (p < 0.05) (Fig. 5A). Higher proportions of CD4+ memory cells were activated in the primigravid group (p < 0.001) and the parous group (p < 0.05) compared to the nulligravid group (Fig. 5B). In the CD4+ CM cell population (p < 0.001) and the CD4+ EM cell population (p < 0.0001) activated proportions were higher in primigravid compared to nulligravid women (Fig. 5C and D). Also, in both the CD4+ CM and EM population a significantly higher proportion of activated cells was found in parous women compared to nulligravid women (p < 0.05) (Fig. 5C and D). The activated proportion of CD4+ EM cells was lower in parous women compared to primigravid women (p < 0.05) (Fig. 5D). Interestingly, contrary to CD4+ memory cells, no differences were observed in activation status of CD8+ memory cells between the groups (Fig. 5E, F, G, and H).

### 4. Discussion

This study shows that pregnancy has short term and persistent effects on CD4+ and CD8+ memory cell subsets in peripheral blood. Firstly, these effects are short term, as shown by the significantly higher CD4+ EM cell and activated CD4+ memory cell proportions in primigravid women, and secondly persistent, as shown by the significantly higher proportions CD4+ and CD8+ EM, CD4+ CM and activated CD4+ memory T cells in parous women.

A higher proportion of CD4+ EM cells was observed during pregnancy and both CD4+ EM and CM cell subsets persisted higher after pregnancy. Our data appears to be in line with other human and murine studies (Gomez-Lopez et al., 2013; Kinder et al., 2014; Piper et al., 2007; Rosenblum et al., 2015, 2011; Rowe et al., 2012; Tiburges et al., 2010), supporting the hypothesis that maternal memory T cell populations are generated during pregnancy and are persistently altered after pregnancy. The mouse model from Rowe et al., shows that CD4+ memory cells are generated during pregnancy and remain at elevated levels afterwards (Rowe et al., 2012). During a subsequent gestation, the memory T cell population rapidly re-expanded and fetal resorption rate was significantly decreased compared to the first pregnancy and compared to fetal specific memory T cell ablated mice (Rowe et al., 2012). The human equivalent and the immunology behind this mouse model is yet to be found, however the current study does support the findings that post-partum memory T cell populations are persistently affected. The effects of these alterations on pregnancy complication rates in subsequent pregnancies should be the focus of future research. Following Rowe et al. and Rosenblum et al., regulatory memory T cells should be the subset to focus on in these future studies (Rosenblum et al., 2015; Rowe et al., 2012).

The higher proportions of CD4+ EM cells found in primigravid women compared with nulligravid women in this study, could be explained by continuous exposure to fetal antigens during pregnancy (Lo et al., 2000; Nelson, 2008; Sunami et al., 2010). However, other explanations include the more activated status of the innate immune system which leads to more antigen presentation by antigen presenting cells and therewith generation of memory T cells (Luppi et al., 2002). Expansion of the EM cell population can occur through two pathways. The first pathway is via dendritic cells carrying antigens that stimulate naive T cells to differentiate to effector cells and thereafter into EM cells (Lanzavecchia and Sallusto, 2005). In the second pathway, CD4+ CM cells convert to CD4+ EM cells upon antigen re-exposure (Sallusto et al., 2004). In parous women we also observed an increased proportion of CD4+ memory cells. In these women, not only the CD4+ EM cells were increased, also the overall memory cell proportion of CD4+ cells and the CM cell proportion are significantly higher in parous women compared to nulligravid women. As explained above, a possible explanation could be the generation of fetal antigen specific memory T cells during pregnancy which are maintained by ongoing antigen-activation by fetal cells that persist as fetal microchimerism (Bianchi et al., 1996; Nelson, 2008). The significant difference in CD4+ EM cell proportions between parous and nulligravid women is less obvious than the difference in CD4+ CM cell proportions between these groups. Possibly, this is due to the fact that CM cell populations are able to renew themselves and therefore are able to remain present at higher levels whereas EM cell populations decrease over time (Lanzavecchia and Sallusto, 2005).

Next to the increased proportions of CD4+ memory cells during pregnancy, the memory T cell populations appeared to be more activated as well, as measured by CD69 expression (Ziegler et al., 1994). The increased proportion of activated CD4+ CM and EM cells found in primigravid women is in accordance with the increased proportion of CD4+ EM cells that was found during pregnancy, since activation of CM cells is essential for the transition to EM cells (Sallusto et al., 2004). Also, significantly higher activation status of the CD4+ memory cell subsets in parous women was found compared to nulligravid women. The activated proportions of the total CD4+ cell population did not differ between these two groups, showing that the difference found between nulligravid and parous women in the memory T cell subsets is specific for memory T cells and is not caused by activation of the total CD4+ cell population. The higher activated proportions of memory T cells in parous women compared to nulligravid women is an interesting observation knowing that next to cytokines that maintain a memory T cell population, continuous activation by antigens through MHC Class II is beneficial for a CD4+ memory cell population to persist and maintain its immunosuppressive properties (Nelson et al., 2013). The known presence of fetal cells in the maternal circulation postpartum, i.e. fetal microchimerism, could be causing the antigen reminders for the memory T cell population and possibly causes the activation shown in this study (Bianchi et al., 1996; Nelson, 2008).
Fig. 4. CD8+ memory cells in nulligravid, primigravid and parous women. Proportions and absolute cell counts of CD8+ memory (CD8+CD45RO+CCR7+) cells (A and D), CD8+ central memory (CM; CD45RO+CCR7+) cells (B and E) and CD8+ effector memory (EM; CD45RO+CCR7−) cells (C and F). Frequencies as proportions of CD8+ cells. Symbols represent individual values per donor and horizontal lines indicate means ± SEM. Analysis by one-way ANOVA followed by Tukey’s post hoc test; *p < 0.05.

Fig. 5. Activated T-lymphocytes in nulligravid, primigravid and parous women. Proportions of activated (CD69+) cells of CD4+ cells (A), CD4+ memory cells (B), CD4+ central memory (CM; CD45RO+CCR7+) cells (C), CD4+ effector memory (EM; CD45RO+CCR7−) cells (D), CD8+ cells (E), CD8+ memory cells (F), CD8+ central memory (CM; CD45RO+CCR7+) cells (G), and CD8+ effector memory (EM, CD45RO+CCR7−) cells (H). Symbols represent individual values per donor and horizontal lines indicate means ± SEM. Analysis by one-way ANOVA followed by Tukey’s post hoc test; *p < 0.05, **p < 0.001, ***p < 0.0001.

Analyses of proportions give insight into processes within the CD4+ and CD8+ cell population in the peripheral blood. The absolute cell counts of the CD4+ memory cell subsets were lower during pregnancy and were back to pre-pregnancy levels in parous women. The clearly lower proportion of T-lymphocytes within the total white blood cells in primigravid women compared to both non-pregnant groups (Fig. 2A) could partly explain the lower memory T cell counts per liter in primigravid women found in this study. The lower proportion of T-lymphocytes within white blood cells is probably due to expansion of the granulocyte population during pregnancy (Luppi et al., 2002). Additionally, it is known that the blood volume in pregnant women is higher compared to nulligravid women and this contributes to lowering the lymphocyte count per liter despite the increased WBC count due to granulocytes (Hyttén and Painthin, 1963; Luppi et al., 2002). A contributing factor for the lower T lymphocyte proportion found in primigravid women could be migration patterns of T cells. In case of an infection, T cells become activated, differentiate and then migrate to the infection site (Sallusto et al., 2004). Possibly, during pregnancy the same process occurs, only instead of migrating to the site of infection, the cells migrate to the site where fetal cells come into contact with maternal cells and are exposed to fetal antigen, i.e.
the fetal maternal interface. Indeed, studies have shown increased CD4+ and CD8+ memory cell proportions in the decidua compared to peripheral blood (Saito et al., 1994; Tilburgs et al., 2010). After pregnancy, migration of the memory T cells to the peripheral blood could be the reason for the increased counts postpartum, possibly representing the latent memory T cell population ready for antigen re-exposure in a following pregnancy.

This study shows higher CD8+ cell proportions of lymphocytes in parous women compared to nulligravid women. This confirms data from Watanabe et al. showing an increase of CD8+ T cells from 1 to 4 months postpartum (Watanabe et al., 1997, 1996). However, contrary to the differences in CD4+ memory cells, the CD8+ memory cell proportions and their activation status appeared to be more comparable between the groups; in primigravid women, no differences in memory T cell populations were found compared with nulligravid women. Mainly CD8+ EM cells are suggested to play a role in the maternal immune response (Lissauer et al., 2012; Tilburgs et al., 2010; Tilburgs and Strominger, 2013; van Egmond et al., 2016; Wang et al., 2015). However, most of the studies supporting the role for CD8+ cells in tolerance found altered CD8+ cell populations in decidua tissue and their specificity remains incompletely understood (Tilburgs et al., 2010; Tilburgs and Strominger, 2013; van Egmond et al., 2016; Wang et al., 2015). In decidua tissue, accumulation of activated CD8+ EM cells in comparison to the peripheral blood was described (Tilburgs et al., 2010; Tilburgs and Strominger, 2013). Differences in the peripheral blood are rarely described in literature. One study reported the presence of fetal specific CD8+ EM cells in the peripheral blood, expansion in magnitude of this population during pregnancy and increase of the fetal specific response postnatally (Lissauer et al., 2012). This may be in line with the current data, since we detected slight, but significantly higher proportions and counts of CD8+ memory cells in parous women. Probably, adaptations of CD8+ memory cells are mainly found locally at the fetal maternal interface and these subsets only differ slightly in peripheral blood. We conclude that further research on CD8+ memory cells should be performed at the fetal maternal interface and not in the peripheral circulation.

The maternal immune response is possibly different in subsequent pregnancies compared to first pregnancies and differs throughout pregnancy. Data from sub analyses, using correlation coefficients, showed that the number of pregnancies that parous women had before blood withdrawal, the period of time after their last pregnancy and weeks of gestation in primigravid women, did not correlate with the proportions and counts of memory T cells. A limitation of this study is the small sample size, however, the patient groups are very homogenous. Firstly, only healthy primigravid women were included in the pregnant group. Secondly, in both healthy non pregnant groups, blood was withdrawn in the first eight days after the start of the menstrual cycle to minimize the effects of sex hormones on the results (Arruvito et al., 2007; Hughes et al., 2013; Lasarte et al., 2013).

Even though a shift from naïve to memory T cells is known to occur with ageing (Globerson and Effros, 2000; Yan et al., 2010), in our study population, including women with marginal age differences, both EM and CM cell populations are not found to increase with age, since linear regression analysis showed that the differences in memory T cell subsets persisted after adjustment for age. Moreover, age was not significantly associated with memory T cell differences and therefore this did not affect our results.

In conclusion, this study shows short and long term effects of pregnancy on memory T cell populations suggesting a shift of CD4+ T cells to EM cells during pregnancy and increased CD4+ CM and EM subsets postpartum. The short and long term effects of pregnancy on the memory T cell populations observed in this study support the hypothesis that memory T cell populations are persistently affected during pregnancy. Whether the persistent alterations of the memory T cell population postpartum is involved in lowering pregnancy complications in subsequent pregnancies and whether these alterations are fetal specific has yet to be investigated.

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