Complications in diabetic pregnancy
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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2014

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

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Chapter three

IMMUNOLOGICAL ADAPTATIONS TO PREGNANCY IN WOMEN WITH TYPE 2 DIABETES.

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Preliminary study.
ABSTRACT

Introduction

Even with adequate glycemic control, pregnancy outcome of women with pregestational type 2 diabetes (T2D) is still less optimal as compared to healthy women. This may suggest that other etiological factors, apart from hyperglycemia, are involved in inducing pregnancy complications. As women with T2D have a proinflammatory state of the immune response, we hypothesized that the immune response of these women may not be able to adapt adequately to pregnancy. To test this hypothesis, we evaluated the immune response of non-pregnant and pregnant women with pregestational T2D and compared this with the immune response of healthy non-pregnant and pregnant women.

Methods

Blood was sampled during the follicular phase in non-pregnant women and around 30 weeks of gestation in pregnant women with and without T2D. The systemic immune response was assessed using flow cytometry in order to evaluate the number and activational status of different lymphocytes, Natural Killer cells and monocyte subsets in the peripheral blood.

Results

A trend towards an increased Th1/Th2 ratio was observed in pregnant women with T2D as compared to healthy women. This was due to a lack of increase in Th2 cells in pregnant women with T2D as compared to non-pregnant women with T2D; in healthy pregnant women, the percentage of Th2 cells increased in pregnant women as compared to non-pregnant women. Cytotoxic NK cells appeared to be more further activated in pregnant women with T2D as compared to healthy pregnant women. Also intermediate and non-classical monocytes appeared to be more activated in pregnant women with type 2 diabetes as compared with healthy pregnant women.

Conclusions

This study showed that pregnancy in women with T2D is associated with an enhanced generalized activation of the innate immune response as compared to healthy pregnancy. Whether such aberrant adaptations of innate immune cells are involved in the development of pregnancy complications remains to be established.
INTRODUCTION

Pregestational type 2 diabetes (T2D) is associated with adverse pregnancy outcome, such as pre-eclampsia, preterm birth, congenital malformations and perinatal death\(^{(1-4)}\). Although hyperglycemia is an important contributor to this adverse outcome and it has been shown that improved glycemic control in women with T2D decreased the number of complications, T2D is still associated with an increased frequency of pregnancy complications\(^{(5)}\). This may suggest that also other etiological factors could be involved. As T2D is associated with immunological alterations, we questioned whether immunological factors may be involved in the development of pregnancy complications in women with pregestational T2D\(^{(6)}\).

Immunological adaptations are needed during pregnancy, in order to facilitate implantation, placentation and tolerance of a semi-allogeneic fetus\(^{(7-9)}\). Such adaptations include a shift towards a type 2 immune response in T-lymphocytes\(^{(7,10)}\) and Natural Killer (NK) cells\(^{(11,12)}\) and an increased frequency of regulatory T cells (Treg) during the first two trimesters of pregnancy\(^{(10,11)}\). Also, activation of monocytes and granulocytes has been observed, suggesting a generalized activation of the inflammatory response\(^{13,16}\). Aberrations in these adaptations are associated with pregnancy complications like recurrent miscarriage, preeclampsia and preterm delivery\(^{(15-17)}\). In view of immunological alteration in women with T2D, the question arises whether these women are able to face these immunological adaptions during pregnancy.

The immunological alterations in patients with T2D are mainly characterized by a chronic and generalized activation of the inflammatory immune response\(^{(6,18)}\). The involvement of the specific immune response is shown by increased numbers of helper type 1 (Th1) and type 17 (Th17) T-lymphocytes and a decreased frequency or function of Treg\(^{(19)}\). As women with T2D have a proinflammatory state of the immune response, we hypothesized that the immune response of these women may not be able to adapt adequately to pregnancy. We tested this hypothesis by evaluating the immune response in the peripheral blood of non-pregnant and pregnant women with and without T2D.

MATERIALS AND METHODS

Patients

This study was registered at the Dutch Trial Register (ID: NTR2195) and approved by the ethic committees of the University Medical Center Groningen (UMCG) and Martini Hospital Groningen (NL30779.042.09). All participants signed informed consent.

Four groups of women (age 18-40 years) were included, i.e. 1) healthy non-pregnant (n=16), 2) healthy pregnant (n=19), 3) non-pregnant with type 2 diabetes (n=6) and pregnant with type 2 diabetes (n=9). Non-pregnant healthy women were recruited from the personnel of the UMCG and healthy pregnant women were recruited from the midwifery clinic of the UMCG. Non-pregnant and pregnant women with pregestational type 2 diabetes were recruited from the diabetes outpatient clinic of the UMCG.
and the Martini Hospital Groningen. Women with type 2 diabetes were defined as diabetic patients who were anti-GAD antibody-negative and/or no occurrence of a keto-acidotic episode and being managed with diet alone or oral blood glucose-lowering agents and/or insulin. All non-pregnant women filled in a questionnaire to obtain information about their cycle, contraception and last menstrual bleeding.

Blood samples were obtained by venous punction into a 10 ml EDTA tube (BD-Plymouth, UK) from non-pregnant women within 10 days after the start of the last cycle and at 30 weeks of gestation in pregnant women. All pregnant women were followed up during pregnancy and 6 weeks postpartum to evaluate pregnancy outcome. For healthy pregnant women, exclusion criteria were gestational diabetes mellitus, known autoimmune, cardiovascular or other active diseases, >2 miscarriages in the past, maternal/fetal complications during pregnancy (i.e. preeclampsia/HELLP, intra-uterine growth restriction, prematurity, perinatal death, congenital malformations and neonatal death). For healthy non-pregnant women, exclusion criteria were known active diseases. For both diabetes groups, exclusion criteria were HbA1c >58 mmol/mol (at the time of sampling) and renal failure (serum creatinine >120 µmol/l).

Outcome measures

The following basic characteristics and pregnancy outcome variables were evaluated (see Table 1 for definitions): Pregestational Body Mass Index (BMI), smoking, alcohol-usage, duration of diabetes, mean HbA1c, parity, preeclampsia/HELLP, prematurity, Caesarean Section (CS), perinatal mortality and macrosomia.

Sample processing and antibodies

The following antibody cocktails were used. Unless stated otherwise, they were purchased from BioLegend (BioLegend Europe, Uithoorn, the Netherlands):

T-lymphocytes antibody cocktail: PerCP labeled anti-human CD3 (clone UCHT1), APC/Cy7 labeled anti-human CD4 (clone OKT4), PE/Cy7 labeled anti-human CD25 (clone BC96), Alexa Fluor 488 labeled anti-mouse/rat/human FoxP3 (clone 150D), Alexa Fluor 488 labeled mouse IgG1 isotype control (clone MOPC-21), PE labeled anti-mouse/human Tbet (clone eBio4B10, eBioscience, Germany), PE labeled anti-mouse/human GATA-3 (clone TWAI, eBioscience) and Alexa Fluor 647 mouse IgG1 isotype control (MOPC-21).

Natural Killer cells antibody cocktail: PerCP labeled anti-human CD3 (clone UCHT1), APC labeled anti-human CD56 (clone MEM188), Pacific Blue labeled anti-human CD16 (clone eBioCB16, eBioscience) and PE labeled anti-human CD335 (clone 9E2), PE labeled mouse IgG1 isotype control (clone MOPC-21).

Monocytes antibody cocktail: PerCP/Cy5.5 labeled anti-human CD14 (clone HCD14), Pacific Blue labeled anti-human CD16 (clone eBioCB16, eBioscience), FITC labeled mouse anti-human HLA-DR, DP, DQ (i.e. MHC-II; clone Tu39, BD Pharmingen, Breda, Netherlands), PE labeled anti-human CD62L (clone DREG-56) and PE labeled mouse IgG1 isotype control (clone MOPC-21).
Reagents

The following reagents were used: Washing buffer (phosphate-buffered saline (PBS) with 0.5% bovine serum albumin and 0.1% NaN3), FACS-TM lysing buffer solution (BD Biosciences, Breda, the Netherlands), FoxP3-staining buffer set (eBioscience, Vienna, Austria) and complete RPMI-1640 medium (Lonza Benelux, Breda, the Netherlands) supplemented with 60 μg/ml gentamycin (Invitrogen, Breda, the Netherlands).

Sample labeling

Processing of blood samples was performed exactly as described before (20). In short, human blood leukocytes were counted, using a microcell counter (Sysmex PocH 100i, Sysmex, Etten-Luer, the Netherlands). Immediately after sampling, whole blood was mixed (1:1) with RPMI-1640 after which the samples were aliquoted into different tubes. After aliquoting, tubes were centrifuged and aspirated. Subsequently, all tubes were incubated with different antibody-cocktails. Subsequently, for all tubes, red blood cells (RBC) were lysed by adding lysing buffer. After centrifugation, aspiration and washing, tubes for lymphocyte/lymphocyte isotype were incubated with fixperm (from FoxP3-staining buffer set). To the other tubes, 300 μl washing-buffer was added; they were then stored in the dark at 4°C until measurement by flow cytometry within 24hr. After incubation in the dark at room temperature, tubes for lymphocyte and lymphocyte isotype staining were washed with 200μl perm (from FoxP3-staining buffer set) followed by incubation with the FoxP3, Tbet and GATA-3 antibodies or their isotype controls and washed with 1 ml perm afterwards. Finally, 300μl washing buffer was added to these tubes and the tubes were stored in the dark at 4°C until measurement by flow cytometry within 24hr.

Flow cytometry

Cells were analysed using the BD LSR II flow cytometer (BD Biosciences, Breda, the Netherlands). At least two hundred fifty thousand events were measured, and data were saved for later analysis. Analyses were performed with FlowJo 7.6.1 (Tree Star, Inc., Ashland, OR, USA).

Data analysis

The protocol for data analysis of the flow cytometry data was described extensively elsewhere (20). An example of the gating strategy for lymphocytes was shown in Figure 1.

Differential cell counts: First, different leukocyte populations (i.e. lymphocytes, monocytes and granulocytes) were assessed. A forward-/sideward scatterplot (FSC/SSC) of the tube stained for NK-cells was standardly used for this purpose. A gate was set around the leukocytes and secondary gates were set around the three different subpopulations in a new FSC/SSC plot.

Lymphocytes: Leukocytes and its subpopulations were identified in a FSC/SSC plot (Fig. 1A,B). These lymphocytes were plotted in a SSC/CD3 graph to identify T-lymphocytes (Fig. 1C). This T-lymphocyte gate was copied to a CD3/CD4 scatterplot to identify Th and Tc (Fig. 1D). A new gate was set in such
a way that CD3- cells were >99% negative for CD25 (i.e. B-lymphocytes, which are CD25-negative (Fig. 1E)). Cells expressing higher levels of CD25 were defined as Teff (Fig. 1F). The gate around Teff was copied to a CD25/FoxP3-plot to identify regulatory T-cells (Treg). In the isotype control, gates for FoxP3 were set in such a way that the positive gate contained no more than 1% of the population (Fig. 1G). This gate was copied to the sample containing the antibody calculating the percentage of Treg (Fig. 1H). Similar gating procedures with isotype controls were used identifying Th1 (CD3+/CD4+/Tbet+) and Th2 (CD3+/CD4+/GATA-3+).

NK-cells: A gate was set around the leukocytes in a FSC/SSC plot. This gate was copied to a new FSC/SSC plot to identify lymphocytes. This gate was then copied to a CD3/CD56 plot and the CD3-/CD56+ (i.e. total NK-cell population) cells were gated to identify the total NK cells. This gate was copied to a CD56/CD16 plot to identify CD56dimCD16++ (i.e. cytotoxic NK (ctNK)) cells and CD56brightCD16+- (i.e. cytokine secreting NK-cells (csNK)). For both subsets, an isotype control was used to assess percentage positive cells and mean fluorescence intensity (MFI) for the marker CD335. The MFI of the positive population was divided by the MFI of the negative population of unstained cells (e.g. MFI CD335-PE+ / MFI unstained for CD335-PE), to correct for day-to-day instrumental variations in fluorescence intensity measurements.
Monocytes: A gate was set around the leukocytes in a FSC/SSC plot and copied to a new FSC/SSC plot, in order to identify monocytes. These cells were moved to a CD14/CD16 plot and divided into three populations, i.e. 1) classical monocytes (CD14hi+/CD16-), 2) intermediate monocytes (CD14++/CD16+) and 3) non-classical monocytes (CD14-/CD16++). For all these subsets, percentage positive cells and MFI of the activation markers CD62L and MHC-II was assessed using isotype controls.
Statistics

Continuous parameters were expressed as mean ± SEM in case of normally distributed data, or as median (Q1-Q3) in case of skewed distribution. To analyse differences in basic characteristics and immunological parameters, independent T-tests or Mann-Whitney U-tests for normally and skewed distributed continuous parameters were used respectively (corrected for multiple testing). Normality of the data was tested using Kolmogorov-Smirnov test. If data were not normally distributed, log-transformation was used. For all analysis, a p-value <0.05 was considered as statistically significant and p-values between 0.05-0.1 were considered as a statistical trend. Statistical analyses were performed using SPSS for windows version 20.0.0.1 (SPSS, Inc., Chicago, IL).

RESULTS

Basic characteristics

Mean age and BMI were significantly higher in pregnant and non-pregnant women with T2D as compared to pregnant and non-pregnant healthy women (Table 1). Due to the small sample size of the T2D groups, no statistical analysis regarding pregnancy complications was performed.

Peripheral immune response

Type 2 diabetic pregnant women showed no different adaptations in the percentage of monocytes, granulocytes and lymphocytes.

The number of leukocytes and their subpopulations were not different between non-pregnant women with and without T2D (Fig. 2). During pregnancy, the number of leukocytes was increased in both pregnant groups as compared to their non-pregnant controls. The number of lymphocytes appeared to decrease in both groups of pregnant women as compared to their respective non-pregnant controls, although this was only significant for healthy women (Fig. 2b). The number of monocytes was comparable between pregnant and non-pregnant women with T2D, while an increased number of these cells was observed in healthy pregnant women as compared to non-pregnant healthy women. However, during pregnancy, the number of monocytes was similar in diabetic and healthy women (Fig. 2c). Granulocyte numbers were not different between non-pregnant T2D and healthy women and increased in both groups during pregnancy (Fig. 2d).

Lymphocytes: Lack of increase in Th2 cells in pregnant women with T2D.

The percentage of T-lymphocytes tended to be higher in non-pregnant women with T2D as compared to healthy non-pregnant women (Fig. 3a). In healthy women, but not in T2D women, an increased percentage of T-lymphocytes was observed during pregnancy; this resulted in a decreased percentage of these cells in pregnant women with T2D as compared to healthy pregnant women. The Th/Tc ratio was decreased in non-pregnant women with T2D vs. healthy controls (Fig. 3b). During pregnancy,
no changes were observed in this ratio in women with T2D, while a significantly increased ratio was found in healthy pregnant women. No differences were found in percentages of naïve T-cells, effector T-cells (Teff) and CD4+CD25+FoxP3+ (Treg) cells between non-pregnant and pregnant women with and without T2D (Fig. 3c-e). The percentage of Tbet positive Th (i.e. Th1) cells was significantly increased in non-pregnant women with T2D vs. healthy controls (Fig. 3f). During pregnancy, the percentage of Th1 cells tended to decrease in women with T2D as compared to non-pregnant women with T2D, while it did not change in healthy women during pregnancy. This resulted in no differences in Th1 cells between both pregnant groups. No differences were found in GATA-3 positive Th (i.e. Th2)
cells between both non-pregnant groups (Fig. 3g). However, in contrast to pregnant women with T2D, an increased percentage of Th2 cells was observed in healthy women during pregnancy. The changes in Th1 and Th2 cells during pregnancy resulted in an increased Th1/T2-ratio (trend) in pregnant women with T2D vs. healthy pregnant women (Fig. 3h).

NK-cells: Increased activational status of cytotoxic NK cells in pregnant women with T2D.

The percentage of NK cells (i.e. all CD56+ cells) was lower in non-pregnant and pregnant women with T2D as compared with non-pregnant and pregnant healthy controls (Fig. 4a). No differences in the ctNK/csNK ratio were found between both non-pregnant groups (Fig. 4b,c). During pregnancy, in both women with and without T2D, the ratio ctNK/csNK cells was decreased as compared to their respective non-pregnant controls, although this was not significant for pregnant women with T2D. Although no differences in the percentage of NKT cells were observed between non-pregnant women with and without T2D, a decreased percentage of NKT cells was found in pregnant women with T2D as compared to healthy pregnant controls.
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Figure 3. The percentage of T-lymphocytes [A], ratio of helper (Th) and cytotoxic (Tc) T-cells [B], percentage of naive T-cells [C], effector T-cells [D], regulatory T-cells (Treg) [E], Tbet positive Th (as a transcription factor for Th1) [F], GATA-3 positive Th (as a transcription factor for Th2) [G] and the ratio Th1/Th2 [H] in non-pregnant women with T2D (T2D NP), pregnant women with T2D (T2D P), healthy non-pregnant women (H NP) and healthy pregnant women (H P). Cell populations were identified based on forward and side scatter. TWA: two-way ANOVA.

*: Significant difference between the pregnant and non-pregnant women, Bonferroni posttest, p<0.05.
To evaluate the activational status of NK cells, the percentage and MFI of the activation marker CD335 was studied. No differences were found in percentage positive cells and MFI of CD335 on ctNK cells between both non-pregnant groups (Fig. 4d,e). During pregnancy, the percentage CD335 positive ctNK cells was significantly increased in both T2D and healthy women. The MFI of CD335 on ctNK cells was increased in both groups of women during pregnancy, however, this was only significant for women with T2D.

The percentage of CD335 positive csNK cells was not affected by diabetes or pregnancy different between both non-pregnant groups nor during pregnancy (Fig. 4f). Although the MFI of CD335 on csNK cells was not different in both non-pregnant and pregnant women with T2D as compared to healthy women, the MFI of CD335 on csNK cells tended to decrease in healthy pregnant women vs.

**Figure 4.** The percentage of total NK cells [A], ratio of cytotoxic (ct)NK cells and cytokine secreting (cs)NK cells [B], NKT cells [C], the percentages and the mean fluorescence intensity (MFI) of CD335 on ctNK [D,E] and csNK cells [F,G] in non-pregnant women with T2D (T2D NP), pregnant women with T2D (T2D P), healthy non-pregnant women (H NP) and healthy pregnant women (H P). Cell populations were identified based on forward and side scatter. TWA: two-way ANOVA.

*: Significant difference between the pregnant and non-pregnant women, Bonferroni posttest, p<0.05.
Monocytes: Increased activation of intermediate monocytes in pregnant women with T2D.

No differences were found in the percentages of the three monocyte subsets (i.e. classical, intermediate and non-classical) between non-pregnant women with T2D in comparison with healthy non-pregnant women (Fig. 5a-c). In healthy pregnant women, a decreased percentage of classical and an increased percentage of intermediate monocytes was observed as compared with healthy non-pregnant women. In pregnant women with T2D, no changes in these monocyte subsets were observed as compared to non-pregnant women with T2D; this resulted in a decreased percentage of intermediate monocytes in T2D pregnant women as compared to healthy pregnant women.

The activational status of the monocyte subsets was evaluated by the activation markers MHC-II and CD62L. In all groups of women, the three monocyte subsets were 100% positive for MHC-II (data not shown). The MFI of MHC-II was similar between both non-pregnant groups in all three monocyte subsets (Fig. 5d-f). During healthy pregnancy, the MFI of MHC-II of classical and intermediate monocytes significantly decreased, while this was not observed in type 2 diabetic women. Although the MFI of MHC II of non-classical monocytes appeared to decrease during healthy and T2D pregnancy, this was not significant.

The percentage of CD62L positive classical monocytes did not differ between non-pregnant women with T2D and healthy non-pregnant women (Fig. 5g). However, in the intermediate and non-classical subsets, an increased percentage of CD62L positive cells was found in non-pregnant women with T2D vs. healthy non-pregnant women (Fig. 5h,i). No effect of pregnancy on percentage of CD62L positive classical monocytes was observed. However, for intermediate and non-classical monocytes, percentage CD62L positive cells decreased during pregnancy in women with T2D, while the percentage of CD62L of these monocyte subsets increased during healthy pregnancy. The MFI of CD62L on classical monocytes was similar between both non-pregnant groups, while a trend to a decreased MFI of CD62L in pregnant women with T2D as compared to non-pregnant women with T2D and no effects of healthy pregnancy on MFI of CD62L on classical monocytes (Fig. 5j). The MFI of this activation marker was increased on intermediate monocytes in non-pregnant women with T2D vs. non-pregnant healthy controls; there was no effect of pregnancy in healthy women, while in T2D women the MFI of CD62L on intermediate monocytes significantly decreased during pregnancy, resulting in a decreased MFI of CD62L on intermediate monocytes in pregnant women with T2D as compared to healthy pregnant women (Fig. 5k). In non-classical monocytes, the MFI of CD62L was decreased in non-pregnant women with T2D vs. healthy non-pregnant women (Fig. 5j). During pregnancy, the MFI of CD62L on non-classical monocytes did not change in women with T2D, but increased in healthy women. Therefore, during pregnancy, the MFI of CD62L on non-classical monocytes was similar in both groups of women.
Figure 5. The percentage of classical, intermediate and non-classical monocytes [A-C], MFI of MHC-II of the three subsets [D-F], and percentage and MFI of CD62L positive monocyte subsets [G-L] in non-pregnant women with T2D (T2D NP), pregnant women with T2D (T2D P), healthy non-pregnant women (H NP) and healthy pregnant women (H P). Cell populations were identified based on forward and side scatter. TWA: two-way ANOVA.

*: Significant difference between the pregnant and non-pregnant women, Bonferroni posttest, p<0.05.
DISCUSSION

This preliminary study showed different adaptations of the immune response to pregnancy in women with T2D as compared to healthy women. Although the percentage of Th1 cells decreased in women with T2D during pregnancy to levels seen in healthy pregnant women, in contrast to healthy pregnant women, the T2D women lacked an increase in Th2 cells. This resulted in a trend towards an increased Th1/Th2 ratio in pregnant women with T2D as compared to healthy pregnant women. Pregnant women with T2D also showed an increased activation of cTNK cells and intermediate and non-classical monocytes as compared to healthy pregnant women. Based on these findings, it may be postulated that the general activation of the innate immune response in women with T2D is further enhanced as compared with healthy pregnancy. Although T-cells do adapt to pregnancy in women with T2D, they may not adapt properly.

It is generally accepted that T2D is associated with a generalized activation of the inflammatory response\(^6\). Whether monocytes in our group of non-pregnant women with T1D are activated is unclear, since we showed opposite results for CD62L expression on the three monocyte subsets. In general, CD62L is shed upon activation of the monocytes\(^21\). However, we observed an increased percentage positive CD62L intermediate and non-classical monocytes in non-pregnant women with T2D vs. healthy women, while the MFI of CD62L on non-classical monocytes was decreased in women with T2D as compared to healthy women. The net result of these changes is unclear at the moment. We also observed an increased percentage of Th1 cells in non-pregnant T2D women, which does suggest of a proinflammatory state. Such a proinflammatory state in T2D women may be due to the increased presence of adipose tissue in women with T2D, since T2D women in our study had a significantly higher BMI as compared to healthy women. This may result in decreased adiponectin levels in these women\(^22\). As adiponectin has anti-inflammatory properties, the decreased levels of this molecule may result in a proinflammatory condition, with activated monocytes/macrophages and increased levels of proinflammatory cytokines like TNF-\(\alpha\), IFN-\(\gamma\), Interleukin-1\(\beta\) and Interleukin-17 as a consequence\(^6,18,23\). This proinflammatory state may lead to insulin resistance\(^6\).

Our data showed that normal pregnancy is associated with a decreased Th1/Th2 ratio, although just not significant, which confirms earlier data\(^7,10\). This was due to an increased percentage of Th2 cells in healthy pregnant women. In T2D pregnancy, we observed a trend towards a decrease in Th1 cells, with no differences in Th2 cells in women with T2D during pregnancy. Although the Th1/Th2 ratio appeared to decrease during pregnancy in these T2D women as well, it still tended to be higher as healthy pregnant women. Such a putative shift towards a Th1 immune response may play a role in pregnancy complications, which can be observed in women with T2D, since a predominant Th1 immune response is associated with adverse pregnancy outcome\(^10,15,24\).

In both pregnant and non-pregnant women with T2D, the number of NK cells was decreased as compared to healthy women. These findings do not agree with those of a recent paper, showing no differences in the frequency of NK cells in patients with T2D as compared to healthy controls\(^25\). This discordance may possibly be explained by the relatively high BMI of the T2D women in our study, since obesity is associated with decreased frequencies of NK cells\(^26\). During pregnancy in both T2D
and healthy women, the ctNK/csNK cell ratio was decreased, although this was only significant in healthy women. A decreased ctNK/csNK cell ratio was observed earlier during pregnancy and may be important for fetal tolerance (27,28). The relative lesser decrease of the ctNK/csNK cell ratio in pregnant women with T2D was associated with an increased expression of CD335 on the ctNK cells suggesting that there are not only relatively more ctNK cells present in a T2D pregnancy, but that they may also be more cytotoxic. This may result in increased cytotoxicity towards fetal trophoblast cells and may negatively affect the placenta (27).

An increased percentage of intermediate monocytes and a decreased percentage of classical monocytes during normal pregnancy is suggestive of a proinflammatory state (29), since previous studies have shown that a relative increase of the intermediate monocytes was associated with inflammatory diseases (29,30). This is in line with the general idea that pregnancy is associated with a generalized inflammatory response (31). Interestingly, in pregnant women with T2D, the percentage of classical monocytes tended to be increased, while the percentage of intermediate monocytes was decreased as compared to healthy pregnant women. This may suggest that there is less activation of the inflammatory response in T2D pregnancy. On the other hand, the finding that CD62L, an adhesion molecule, was decreased in both intermediate and non-classical monocytes in T2D pregnancy as compared to healthy pregnant women and non-pregnant T2D women, may suggest that there is increased activation of these monocyte subsets in T2D during pregnancy. Although further studies into the monocytes in T2D are warranted, our data seem to suggest further activation of the monocytes in T2D pregnancy.

The present study showed aberrant adaptations of the immune response to pregnancy in T2D in comparison with healthy women. Although we also observed aberrant adaptations of the immune response to pregnancy in women with type 1 diabetes (T1D) (20), there are differences in pregnancy induced immunological adaptations between T2D and T1D. In general, in T1D, important differences in adaptation were found in lymphocytes, NK cells and monocytes, while in T2D main differences in adaptation were found in NK cells and monocytes only. Although the percentage of Th1 cells was increased in both non-pregnant women with T1D and T2D, the percentage of Th1 cells was only decreased during pregnancy in women with T2D. This was in accordance with adaptations during healthy pregnancy (7,10). In pregnant women with T1D, however, the percentage Th1 cells was even further increased during pregnancy as compared with non-pregnant women with T1D (20). It thus appears that, women with T1D, being a Th1 type autoimmune disease, are not able to down regulate the number of Th1 during pregnancy, while women with T2D are able to decrease Th1 cells to a certain extend. These different adaptations should possibly be explained by the etiological differences between both diseases and suggest intrinsic differences in Th1 lymphocytes between T1D and T2D. Interestingly, while the innate immune response showed similar changes in non-pregnant women with T1D and T2D as compared to healthy non-pregnant women, the adaptations during pregnancy in these 2 types of diabetes are also very similar. This may suggest that the characteristics of innate immune cells before pregnancy determine how they adapt to pregnancy.

In conclusion, this study showed that pregnancy in women with T2D is associated with an enhanced generalized activation of the innate immune response as compared to healthy pregnancy. Whether
such aberrant adaptations of innate immune cells are involved in the development of pregnancy complications remains to be established. However, since it is generally accepted that aberrant immunological adaptations to pregnancy are associated with pregnancy complications\cite{15-17}, it is tempting to speculate that the observed enhanced generalized activation of the immune response in pregnant women with diabetes mellitus is (in addition to hyperglycemia) involved in the development of pregnancy complications. Additional studies in order to accomplish further etiological insights into the development of pregnancy complications in women with T2D are needed.
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