Clinical & Experimental Immunology
The Journal of Translational Immunology

Clinical and Experimental Immunology ORIGINAL ARTICLE doi:10.1111/cei.12300

The role of regulatory T cell (T_{reg}) subsets in gestational diabetes mellitus

L. Schober,* D. Radnai,* J. Spratte,* A. Kisielewicz,* E. Schmitt,† K. Mahnke,‡ H. Fluhr,* L. Uhlmann,§ C. Sohn* and A. Steinborn*

*Departments of *Obstetrics and Gynecology, ‡Dermatology and §Institute of Medical Biometry and Informatics, University of Heidelberg, Heidelberg, and †Institute of Immunology, University of Mainz, Mainz, Germany*

Accepted for publication 13 February 2014
Correspondence: A. Steinborn, Department of Obstetrics and Gynecology, University of Heidelberg, INF 440, 69120 Heidelberg, Germany.
E-mail: andrea.steinborn@med.uni-heidelberg.de

Summary

Physiological changes during normal pregnancy are characterized by an inflammatory immune response and insulin resistance. Therefore, we hypothesize that gestational diabetes mellitus (GDM) may be caused by an inappropriate adaption of the maternal immune system to pregnancy. In this study we examined the role of regulatory T cell (T_{reg}) differentiation for the development of GDM during pregnancy. We used six-colour flow cytometric analysis to demonstrate that the total $CD4^+CD127^{low+/-}CD25^+$ forkhead box protein 3 ($FoxP3^+$) T_{reg} pool consists of four different T_{reg} subsets: naive $CD45RA^+$ T_{regs}, $HLA\text{-}DR^-CD45RA^-$ memory T_{regs} (DR^- T_{regs}) and the highly differentiated and activated $HLA\text{-}DR^{low+}CD45RA^-$ and $HLA\text{-}DR^{high+}$ $CD45RA^-$ memory T_{regs} (DR^{low+} and DR^{high+} T_{regs}). Compared to healthy pregnancies, the percentage of $CD4^+CD127^{low+/-}CD25^+FoxP3^+$ T_{regs} within the total $CD4^+$ T helper cell pool was not different in patients affected by GDM. However, the suppressive activity of the total $CD4^+CD127^{low+/-}CD25^+$ T_{reg} pool was significantly reduced in GDM patients. The composition of the total T_{reg} pool changed in the way that its percentage of naive $CD45RA^+$ T_{regs} was decreased significantly in both patients with dietary-adjusted GDM and patients with insulin-dependent GDM. In contrast, the percentage of DR^--memory T_{regs} was increased significantly in patients with dietary-adjusted GDM, while the percentage of DR^{low+} and DR^{high+} memory T_{regs} was increased significantly in patients with insulin-dependent GDM. Hence, our findings propose that alterations in homeostatic parameters related to the development and function of naive and memory T_{regs} may cause the reduction of the suppressive capacity of the total T_{reg} pool in GDM patients. However, as this is an exploratory analysis, the results are only suggestive and require further validation.

Keywords: pregnancy, immune suppression, subsets of regulatory T cells, gestational diabetes mellitus, inflammation

Introduction

Gestational diabetes mellitus (GDM) is defined as carbohydrate intolerance with onset or first recognition during pregnancy. It represents a common metabolic complication that affects about 2–9% of all pregnant women in western countries [1]. The resulting maternal hyperglycaemia is associated with numerous adverse neonatal outcomes, such as neonatal hypoglycaemia and macrosomia, and therefore with an increased risk of shoulder dystocia. Maternal complications primarily include an increased risk of pre-eclampsia [2]. Furthermore, women with GDM have an increased risk of developing type 2 diabetes mellitus within 5–15 years of delivery [3], while children exposed prenatally to a diabetic milieu also have an increased risk for the development of type 2 diabetes during later life [4]. Currently, GDM screening tests are used to identify women with GDM [5], who are normally treated either with diet or insulin. Despite the better detection of GDM and subsequent therapy, the underlying pathophysiology of GDM is largely unknown. Similar to patients with type 2 diabetes, GDM patients are unable to increase insulin production to

compensate for the increased insulin resistance during pregnancy [6]. Therefore, it is assumed that women developing GDM already suffer from chronic reduced insulin sensitivity before conception that is enhanced further by the physiological insulin resistance occurring after 20 weeks of gestation [7]. Recently, several susceptible genes involved in impaired β cell function, insulin resistance and abnormal glucose utilization were shown to increase the risk of type 2 diabetes mellitus [8]. Meanwhile, these novel gene variants were also detected in GDM patients [9]. However, further genes participating in cell functions involving cell activation, immune response, organ development and regulation of cell death were also found to be expressed differentially in GDM patients [10]. In addition to exaggerated glucose intolerance and insulin resistance, GDM is characterized by chronic systemic inflammation [11,12] and an increased humoral immune response [13]. Moreover, recent studies document an important role for adipose tissue inflammasome activation in the development of insulin resistance in GDM pregnancies [14].

In particular, as the immunosuppressive regulatory T cells (T_{regs}) were shown to control excessive inflammation and immoderate immune responses [15] and beyond are known to be of vital importance for the successful course of pregnancy [16], it could be possible that functional deficiencies of such cells are involved in the pathogenesis of GDM. Meanwhile, it is known that the total T_{reg} pool, currently characterized as $CD4^+CD127^{low+/-}CD25^+$forkhead box protein 3 ($FoxP3^+$) T_{reg} cells, consists of less suppressive, naive $CD45RA^+$ T_{regs} and strongly suppressive, antigen-experienced $CD45RO^+$ memory T_{regs} [17]. Thereby, the $CD45RO^+$ T_{reg} population comprises highly differentiated human leucocyte antigen D-related (HLA-DR)-expressing cells, which were shown to reveal higher FoxP3 expression levels and to possess stronger suppressive activity than $HLA\text{-}DR^-$ memory T_{regs} [18]. Recently, our group demonstrated that the total $CD4^+CD127^{low+/-}CD25^+FoxP3^+$ T_{reg} cell pool can be divided into four distinct T_{reg} subsets: naive $CD45RA^+$ T_{regs}, $HLA\text{-}DR^-CD45RA^-$ memory T_{regs} (DR^- T_{regs}), $HLA\text{-}DR^{low+}CD45RA^-$ memory T_{regs} (DR^{low+} T_{regs}) and $HLA\text{-}DR^{high+}CD45RA^-$ memory T_{regs} (DR^{high+} T_{regs}). The composition of the total T_{reg} pool with these distinct subsets changed both during the normal course of pregnancy and in the presence of characteristic gestation-associated diseases [19,20]. Thereby, we demonstrated that the ratio of naive $CD45RA^+$ T_{regs} increased during the normal course of pregnancy, but was reduced strongly in the presence of complications such as pre-eclampsia and preterm labour. In contrast, the ratio of memory T_{regs}, especially of the mature $HLA\text{-}DR^{low+}$ and DR^{high+} T_{regs}, was found to be increased in patients affected by pre-eclampsia, while the ratio of DR^- and DR^{low+} T_{regs} was increased in patients affected by preterm labour. Although both forms of diseases revealed lower ratios of the less suppressive $CD45RA^+$-naive T_{regs} and higher ratios of the well suppressive $CD45RA^-$ memory T_{regs}, the suppressive activity of the total T_{reg} pool was reduced significantly in these patients [19,20]. Therefore, it is conceivable that defective T_{reg} cell differentiation, producing less suppressive $CD45RA^-$ memory T_{regs}, may be causatively involved in the development of gestation-associated diseases.

Currently, little information exists about the influence of the immune system on the pathogenesis of GDM. In this study, we demonstrate that the occurrence of GDM is associated with a moderate decrease of its suppressive activity while the size of the $CD4^+CD127^{low+/-}CD25^+FoxP3^+$ T_{reg} pool among the $CD4^+$ T cells is not reduced. Thereby, we also observed characteristic changes in its composition with the above-described T_{reg} subsets: compared to healthy pregnancies, patients with dietary-adjusted GDM had significantly increased percentages of $HLA\text{-}DR^-$ memory T_{regs}, while patients diagnosed with insulin-dependent GDM had increased percentages of activated DR^{low+} and DR^{high+} memory T_{regs}. Both patient groups exhibit decreased percentages of naive $CD45RA^+$ T_{regs}. As this study is an exploratory analysis, the study is only suggestive and needs further validation.

Methods

Patient collectives and healthy volunteers

Blood samples were obtained from 64 healthy pregnant women between 24 and 41 weeks of gestation (group 1), 21 pregnant women with dietary-adjusted gestational diabetes (group 2) and 40 pregnant women with insulin-dependent gestational diabetes (group 3). Table 1 summarizes the clinical characteristics of all participants (groups 1–3). Blood samples from healthy pregnancies were collected from women who had routine ultrasonography to exclude fetal malformations and from women delivering by term elective caesarean section, due to breech presentation, cephalopelvic disproportion or status after caesarean section (group 1). Blood samples from women with gestational diabetes were obtained between 24 and 41 weeks of gestation (groups 2 and 3). The diagnosis of gestational diabetes was made between 24 and 28 weeks of gestation by a positive 2-h 75g oral glucose tolerance test (OGTT) with the following criteria: a fasting plasma glucose ≥ 5·1 mmol/l (92 mg/dl), or a 1-h plasma glucose level of ≥ 10·0 mmol/l (180 mg/dl), or a 2-h plasma glucose of ≥ 8·5 mmol/l (153 mg/dl) [21]. The blood glucose levels of all affected women were checked regularly to determine the individually needed insulin dose. The insulin regimen included an individual combination of short-acting Actrapid® before meals and Protaphane® as long-acting insulin at night. The study was approved by the Regional Ethics Committee. All women were fully informed of the aim of the study and informed consent was obtained from all participants.

Table 1. Clinical characteristics of healthy pregnancies and women with gestational diabetes mellitus (GDM).

	Group 1: healthy pregnancies median (range)	Group 2: Dietary-adjusted GDM median (range)	Group 3: insulin-dependent GDM median (range)
n	64	21	40
Weeks gestation	37 (24–41)	39 (24–41)	36 (24–42)
Age	31 (21–44)	32 (25–43)	34 (22–43)
Weight before pregnancy (kg)	66 (50–117)	67 (45–126)	89 (54–153)
Weight at delivery (kg)	81 (59–128)	83 (58–136)	100 (54–160)
Time since diagnosis (weeks)	–	11 (1–15)	7 (1–15)
Period of treatment (weeks)	–	11 (1–15)	5 (1–12)

For all participants, both the percentage of $CD4^+$ $CD127^{low+/-}CD25^+FoxP3^+$ T_{regs} of total $CD4^+$ T cells and the percentage of naive $CD45RA^+$ T_{regs}, DR^-, DR^{low+} and DR^{high+} memory T_{regs} among the total $CD4^+CD127^{low+/-}CD25^+$ $FoxP3^+$ T_{reg} cell pool were determined by six-colour flow cytometric analysis. For a total number of 33 healthy pregnancies between 24 and 41 weeks of gestation (group 1) and 23 pregnancies with gestational diabetes mellitus (groups 2 and 3), suppression assays were performed in order to evaluate the suppressive capacity of the magnetically selected total $CD4^+CD127^{low+/-}CD25$ T_{reg} cell pool.

Fluorescence-activated cell sorter (FACS) staining

Venous blood samples (10 ml) from all participants were collected into ethylenediamine tetraacetic acid (EDTA)-containing tubes. Whole peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque (Amersham Bioscience, Glattbrugg, Switzerland) gradient centrifugation and analysed by six-colour flow cytometric analysis. Briefly, PBMCs (4×10^6 cells) were surface-stained with allophycocyanin-cyanin 7 (APC-Cy7)-conjugated-anti-CD4 (BD Bioscience, San Jose, CA, USA), peridinin chlorophyll (PerCP)-Cy5·5-conjugated anti-CD127 (BD Bioscience), phycoerythrin (PE)-conjugated anti-CD25 (BD Bioscience), PE-Cy7-conjugated anti-HLA-DR (BD Bioscience) and APC-conjugated anti-CD45RA (BD Bioscience) mouse monoclonal antibodies. Subsequently, intracellular staining for the detection of FoxP3 was performed using a fluorescein isothiocyanate (FITC) labelled anti-human FoxP3 staining set (clone PCH101; eBioscience), according to the manufacturer's instructions. Both the percentage of $CD4^+CD127^{low+/-}CD25^+FoxP3^+$ T_{reg} cells and the percentages of the four T_{reg} subsets (naive $CD45RA^+$ T_{regs}, $HLA\text{-}DR^-$, $HLA\text{-}DR^{low+}$ and $HLA\text{-}DR^{high+}$ memory T_{regs}) within the total T_{reg} pool and the HLA-DR mean fluorescence intensity (MFI) of the $HLA\text{-}DR^+$ T_{reg} subset were determined for all participants. Thereby, the $HLA\text{-}DR^-$ and $HLA\text{-}DR^+$ memory T_{reg} subsets were characterized as $HLA\text{-}DR^-CD45RA^-$ or $HLA\text{-}DR^+CD45RA^-$ memory T_{regs} and abbreviated as DR^-, DR^{low+} and DR^{high+} memory T_{regs}. Negative control samples were incubated with isotype-matched antibodies. Dead cells were excluded by forward- and side-scatter characteristics. Cells were analysed by a FACSCanto cytometer (BD Bioscience). Statistical analysis was based on at least 100 000 gated $CD4^+$ T cells.

Positive selection and staining of $CD4^+CD127^{low+/-}CD25^+$ T_{reg} cells

Whole peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood (50 ml) drawn in EDTA tubes by Ficoll-Hypaque (Amersham Bioscience) gradient centrifugation. $CD4^+CD127^{low+/-}CD25^+$ T_{regs} were purified using the $CD4^+CD127^{low+/-}CD25^+$ Regulatory T Cell Isolation Kit II (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer's instructions. First, $CD4^+CD127^{low+/-}$ T cells were isolated by magnetic depletion of non-$CD4^+CD127^{high+}$ T cells. In a second step, the $CD4^+CD127^{low+/-}CD25^+$ T_{reg} cells were isolated by positive selection over two consecutive columns. The $CD4^+CD127^{low+/-}CD25^-$ T cells were obtained in the flow-through fraction and used as responder T cells (T_{resp}). The $CD4^+CD127^{low+/-}CD25^+$ T_{reg} cells were subsequently retrieved from the columns. The purified $CD4^+CD127^{low+/-}CD25^+$ T_{reg} cell fraction was analysed using four-colour flow cytometry. Briefly, 1×10^5 cells were stained with 10 µl PerCP-conjugated-anti-CD4, PE-conjugated anti-CD25, FITC-conjugated FoxP3 and biotin-conjugated anti-CD127 mouse monoclonal antibodies. Positive staining for CD127 was detected using APC-conjugated streptavidin molecules. On average, 85% of the isolated $CD4^+CD127^{low+/-}CD25^+$ T_{reg} cells were shown to be within the $CD4^+CD127^{low+/-}CD25^+FoxP3^+$ T_{reg} cell population.

Co-culture suppression assay

Whole peripheral mononuclear cells (PBMCs) were isolated from peripheral blood drawn in EDTA tubes by Ficoll-Hypaque (Amersham Bioscience) gradient centrifugation. $CD4^+CD127^{low+/-}CD25$ T_{reg} cells were purified using the $CD4^+CD127^{low+/-}CD25^+$ Regulatory T cell Isolation Kit II (Miltenyi Biotec) described above. In all assays, 2×10^4 responder T cells (T_{resp}) were co-cultured with the purified

$CD4^+CD127^{low+/-}CD25^+$ T_{reg} cells at ratios 1:1–1:64 in 96-well U-bottomed plates. Suppression assays were performed in a final volume of 100 µl/well of X-VIVO15 medium (BioWhittaker, Radnor, PA, USA). For T cell stimulation, the medium was supplemented with 1 µg/ml anti-CD3 and 2 µg/ml anti-CD28 antibodies (eBioscience, San Diego, CA, USA). As controls, $CD4^+CD127^{low+/-}CD25^+$ T_{reg} cells and T_{resp} cells alone were cultured both with and without any stimulus. Cells were incubated at 37°C and 5% of CO_2. After 4 days, 1 µCi [^{3}H]-thymidine was added to the cultures and cells were incubated further for 16 h. Cells were then harvested and [^{3}H]-incorporation was measured by scintillation counting. All assays exhibited < 10% standard error of the mean (s.e.m.) and were performed a minimum of six times. In order to compare the suppressive capacity of the isolated $CD4^+CD127^{low+/-}CD25^+$ T_{regs} between the different patient groups, we calculated the maximum suppressive activity (ratio of T_{reg} cells to T_{resp} cells 1:1). In addition, we determined the suppressive activity of the isolated T_{reg} cells with gradient ratios of T_{reg} cells to T_{resp} cells (ratio of T_{reg} cells to T_{resp} cells 1:1–1:64) and identified the ratio with which a minimum suppression of at least 15% could be achieved (ratio $T_{reg}/T_{resp)}$ [20].

Statistical analysis

As all data were not distributed normally, the statistical comparison of the percentages of $CD4^+$ T cells within total leucocytes, $CD4^+CD127^{low+/-}CD25^+FoxP3^+$ T_{reg} cells within total $CD4^+$ T cells, the different T_{reg} subsets (DR^{low+} T_{regs}, DR^{high+} T_{regs}, DR^- T_{regs} and naive $CD45RA^+$ T_{regs}) within the total $CD4^+CD127^{low+/-}CD25^+FoxP3^+$ T_{reg} cell pool and of its HLA-DR mean fluorescence intensities (MFIs) between the different patient populations (healthy control group, dietary-treated group, insulin-treated group) was performed using the non-parametric Kruskal–Wallis H test. This test is used for simultaneous comparison of more than two sample populations. In addition, dietary- and insulin-treated GDM groups were pooled and compared with the healthy control group using the Wilcoxon–Mann–Whitney *U*-test. Comparison of the suppressive activity of purified $CD4^+CD127^{low+/-}CD25^+$ T_{reg} cells was also performed using the Kruskal–Wallis H test. Each H test was followed by a Dunn test. $P < 0{\cdot}05$ was considered significant. As this was an exploratory analysis, no adjustments for multiple testing were performed.

Results

Gestational diabetes is not associated with deviations in the percentage of $CD4^+CD127^{low+/-}CD25^+FoxP3^+$ T_{reg} cells within the total $CD4^+$ T cell pool

In this study, peripheral blood mononuclear cells (PBMCs) were obtained from 64 healthy pregnancies (group 1), 21 patients with dietary-adjusted GDM (group 2) and 40 patients with insulin-dependent GDM (group 3) (Table 1). Their PBMCs were stained with CD4-, CD127-, CD25-, FoxP3-, HLA-DR- and CD45RA-specific monoclonal antibodies and analysed by six-colour flow cytometric analysis. Figure 1 shows the gating strategy for these measurements. First, $CD4^+$ T cells (Fig. 1a, P1) were analysed for their simultaneous expression of CD25, CD127 and FoxP3 (Fig. 1b, P2 and Fig. 1c, P3). The percentage of $CD4^+CD127^{low+/-}CD25^+FoxP3^+$ T_{reg} cells (Fig. 1c, P3) within the total $CD4^+$ T cell pool, their composition with four distinct T_{reg} subsets [DR^{high+} (P4), DR^{low+} (P5), DR^- memory T_{regs} (P6), naive $CD45RA^+$ T_{regs} (P7)] and the HLA-DR MFI of the DR^+ memory T_{reg} subset (P8) were estimated for all participants (Fig. 1d). Figure 1e,f shows the dot-plots of HLA-DR *versus* CD45RA analysis of one representative experiment performed with PBMCs from a patient with dietary-adjusted GDM (group 2, Fig. 1e) and a patient with insulin-dependent GDM (group 3, Fig. 1f).

We found no significant differences in either the percentage of $CD4^+$ T cells of total leucocytes or the percentage of $CD4^+CD127^{low+/-}CD25^+FoxP3^+$ T_{reg} cells within the total $CD4^+$ T cell pool between healthy pregnancies and pregnancies affected by dietary-adjusted or insulin-dependent GDM (Fig. 2a,b).

In comparison to healthy pregnancies, the suppressive activity of the total $CD4^+CD127^{low+/-}CD25^+$ T_{reg} cell pool is moderately decreased in pregnancies affected by GDM

In order to compare the suppressive capacity of the total $CD4^+CD127^{low+/-}CD25^+$ T_{reg} pool between healthy pregnancies (group 1) and patients with GDM (groups 2 and 3), we performed co-culture suppression assays, as described recently [20]. For that, $CD4^+CD127^{low+/-}CD25^+$ T_{reg} cells were isolated from 33 healthy pregnant women between 24 and 41 weeks of gestation (group 1) and 23 pregnant women affected by GDM (groups 2 and 3) by magnetic affinity cell sorting (MACS) technology. To evaluate the suppressive capacity of the isolated $CD4^+CD127^{low+/-}CD25^+$ T_{regs}, we determined the maximum suppressive activity (Fig. 3a: ratio of T_{reg} cells to T_{resp} cells 1:1) and calculated the ratio of T_{reg} cells to T_{resp} cells with which a minimum suppressive activity of at least 15% could be achieved (Fig. 3b: ratio T_{reg}/T_{resp}). Table 2 and Fig. 3 show the results for these measurements. There were no differences concerning the maximum suppressive activity of the total $CD4^+CD127^{low+/-}CD25^+$ T_{reg} pool (Fig. 3a) between healthy pregnancies (group 1) and pregnancies affected by GDM (groups 2 and 3). However, the ratio of T_{reg}/T_{resp} (Fig. 3b) was decreased significantly ($P < 0{\cdot}01$) in patients with GDM (median 1:4) (groups 2 and 3) compared to healthy pregnancies (median 1:16).

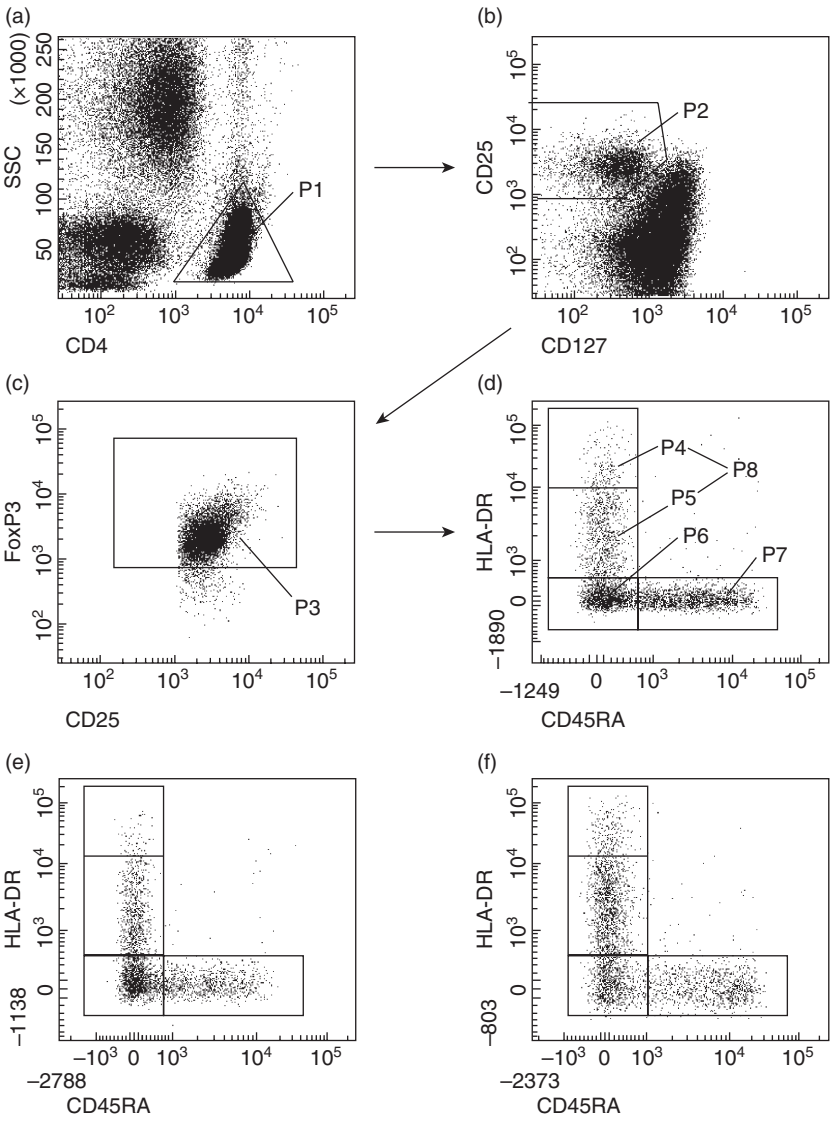

Fig. 1. Gating strategy for six-colour flow cytometric detection of the total $CD4^+CD127^{low+/-}CD25^+$ forkhead box protein 3 ($FoxP3^+$) regulatory T cell (T_{reg}) pool and its percentage of DR^{low+}, DR^{high+}, DR^- memory T_{regs} and naive $CD45RA^+$ T_{regs}. (a) First, $CD4^+$ T cells (P1) were gated by fluorescence intensity of CD4 *versus* side light scatter (SSC). (b) $CD4^+CD127^{low+/-}CD25^+$ T_{reg} cells were gated by fluorescence intensity of CD25 *versus* CD127 (P2). (c) $CD4^+CD127^{low+/-}CD25^+FoxP3^+$ T_{reg} cells were gated by excluding cells without FoxP3 expression (P3). (d) The percentage of the DR^{high+} (P4), the DR^{low+} (P5), the DR^- (P6), and the naive $CD45RA^+$ T_{reg} subset (P7) was estimated by analysing $CD4^+CD127^{low+/-}CD25^+FoxP3^+$ T_{reg} cells (P3) for their expression of human leucocyte antigen D-related (HLA-DR) and CD45RA. Additionally, the HLA-DR mean fluorescence intensity (MFI) of the DR^+ T_{reg} subset (P8) was determined for all participants. (a–d) Representative experiment for healthy pregnancies; (e,f) representative experiments for patients with dietary-adjusted gestational diabetes mellitus (GDM) (e) and with insulin-dependent GDM (f).

Table 2. Percentage of the $CD4^+CD127^{low+/-}CD25^+$ forkhead box protein 3($FoxP3^+$) regulatory T cell (T_{reg}) cell pool within $CD4^+$ T cells, its suppressive activity and its composition with distinct T_{reg} subsets in healthy pregnancies and women with gestational diabetes mellitus (GDM).

	Group 1: healthy pregnancies median (range)	Group 2: dietary-adjusted GDM median (range)	Group 3: insulin-dependent GDM median (range)
$CD4^+$ T cells	32·0 (18·8–49·5)	32·9 (12·7–46·3)	31·2 (13·3–51·2)
T_{reg} cells (%)	5·1 (3·3–8·5)	5·1 (3·9–6·3)	4·8 (2·3–7·9)
Suppressive activity			
Max. suppr. activity (%) (T_{reg}/T_{resp} 1/1)	73·2 (35·8–93·8)	74·0 (0–96·4)	
Ratio of T_{reg} (> 15% suppr. activity)	1:16 (1:2–1:32)	1:4 ↓ (0–1:32)	
Subsets of the T_{reg} pool (%):			
DR^+ T_{regs}	22·8 (12·5–34·5)	25·6 (16·5–41·4)	28·8 ↑ (17·2–56·7)
DR^{low+} T_{regs}	20·3 (11·1–30·7)	22·0 (15·1–35·1)	24·3 ↑ (16·1–46·4)
DR^{high+} T_{regs}	2·5 (0·8–5·2)	2·9 (1·3–6·7)	3·7 ↑ (1·4–15·1)
DR^- T_{regs}	29·9 (13·7–44·8)	34·3 ↑ (24·1–44·9)	31·1 (21·8–44·7)
$CD45RA^+$ T_{regs}	44·4 (25·6–68·9)	35·8 ↓ (20·9–49·0)	36·8 ↓ (8·7–54·5)
MFI			
DR^+ T_{regs}	5047·5 (3033–9053)	4786 (3576–7228)	6424·5 ↑ (3786–11207)

MFI = mean fluorescence intensity; ↑, ↓ = significant differences ($P < 0·05$) were obtained compared to healthy pregnancies. T_{resp} = responder T cells.

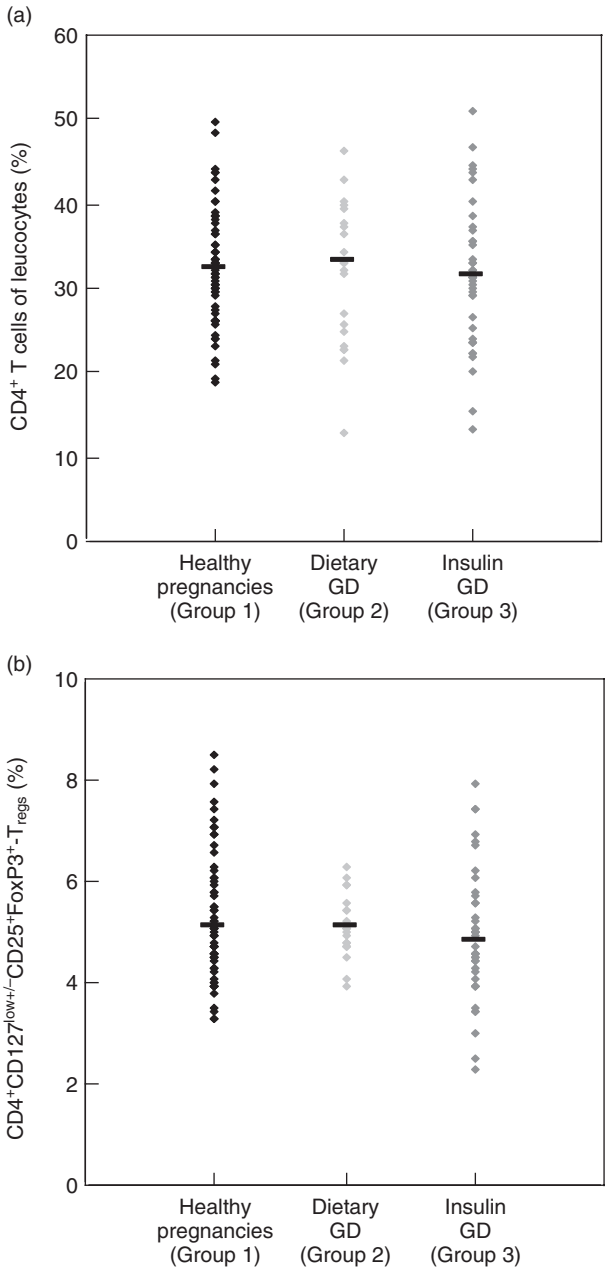

Fig. 2. Detection of the percentage of $CD4^+$ T cells of total leucocytes and the percentage of $CD4^+CD127^{low+/-}CD25^+$ forkhead box protein 3 ($FoxP3^+$) regulatory T cells (T_{regs}) within total $CD4^+$ T cells for healthy pregnancies and patients with dietary-adjusted or insulin-dependent gestational diabetes mellitus (GDM). The figure shows the individual and median data obtained for healthy pregnant women (group 1, ♦), pregnancies affected by dietary-adjusted GDM (group 2◈) and pregnancies with insulin-dependent GDM (group 3◆).

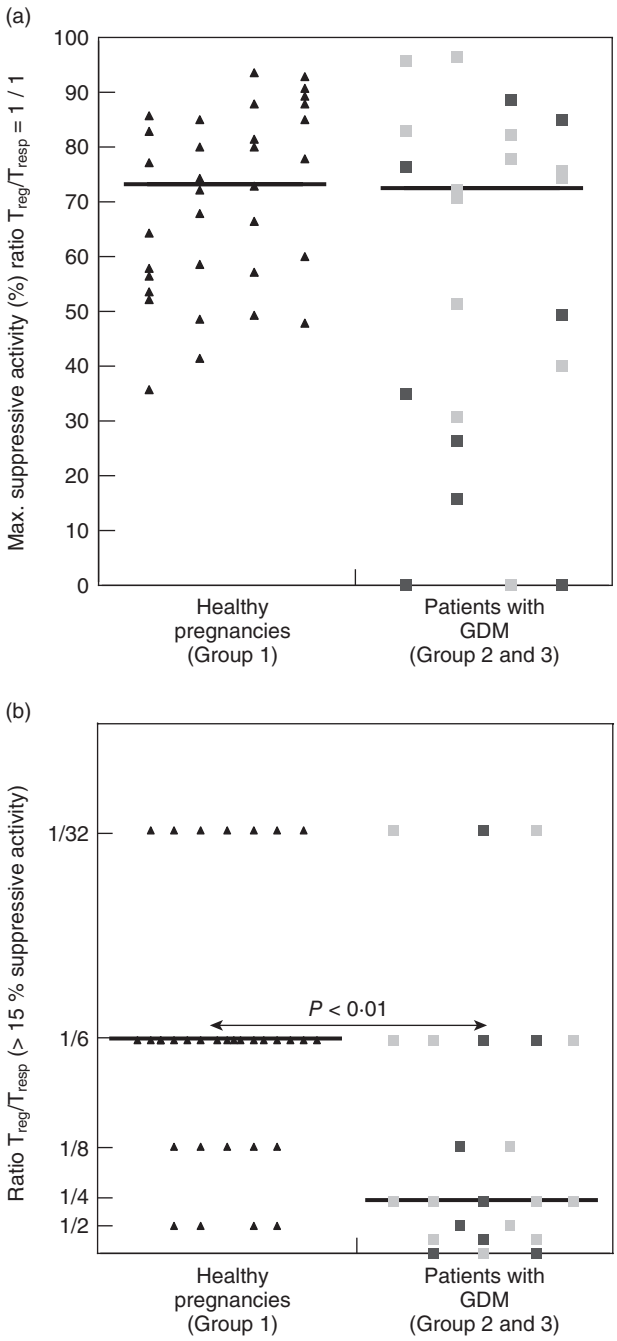

Fig. 3. Evaluation of the suppressive activity of the total $CD4^+CD127^{low+/-}CD25^+$ regulatory T cell (T_{reg}) pool in healthy pregnancies and pregnant women affected by gestational diabetes mellitus (GDM). The total $CD4^+CD127^{low+/-}CD25^+$ T_{reg} pool was isolated using the magnetic-activated cell sorting (MACS) technique and its suppressive activity was examined using suppression assays (see Methods). The figure shows the maximum suppressive activity (ratio $T_{reg}/T = 1/1$), (a) and the ratio of T_{reg}/responder T cells (T_{resp}) up to which the purified T_{regs} could be diluted to achieve a minimum suppressive activity of at least 15% (b). The diagrams represent the individual data obtained for healthy pregnancies (group 1, ▲), for pregnant women with dietary-adjusted GDM (group 2, ◈) and for pregnant women with insulin-dependent GDM (group 3, ■).

The composition of the total $CD4^+CD127^{low+/-}CD25^+$ $FoxP3^+$ T_{reg} cell pool of patients with GDM is different compared to healthy pregnancies

To examine whether the diminished suppressive activity in patients with GDM is associated with changes in the com-

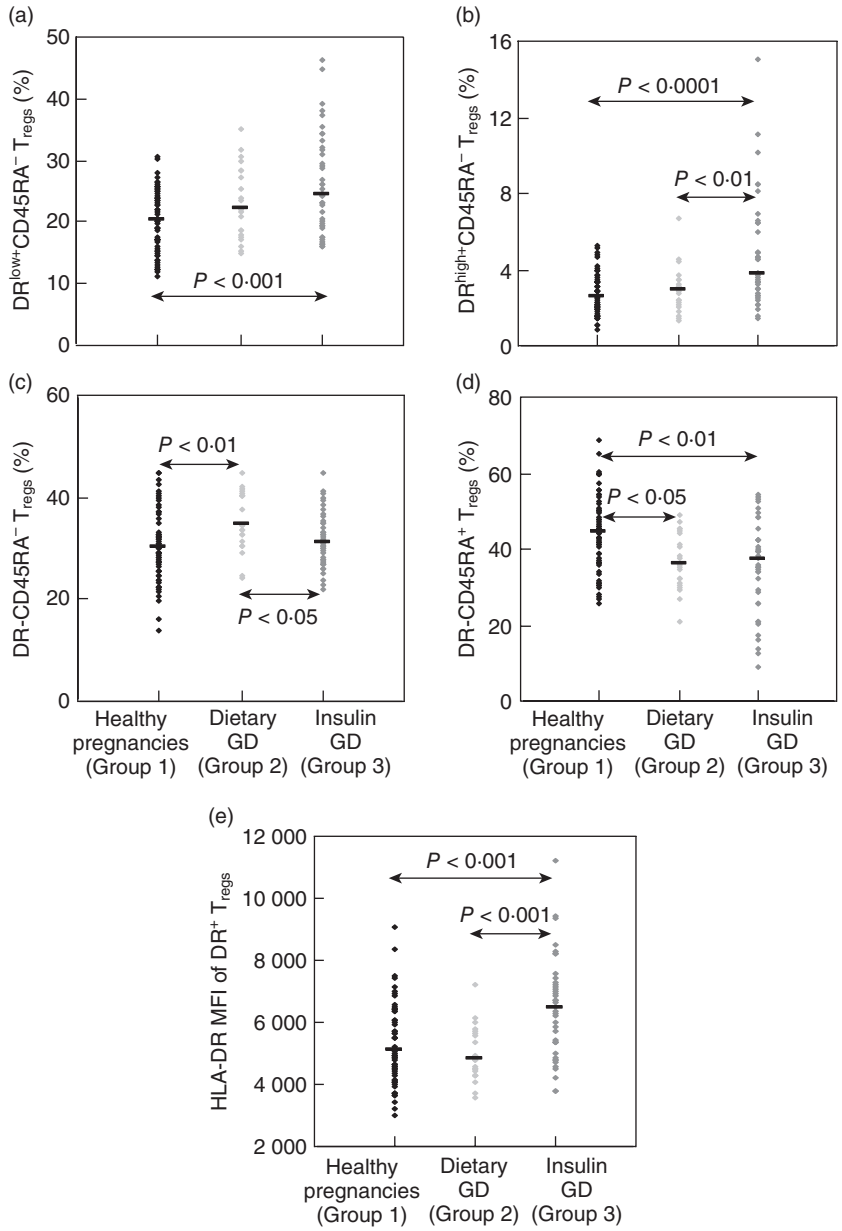

Fig. 4. Detection of the percentages of the DR^{low+}, DR^{high+}, DR^- and $CD45RA^+$ regulatory T cell (T_{reg}) subsets within total $CD4^+CD127^{low+/-}CD25^+$ forkhead box protein 3 ($FoxP3^+$) T_{regs} and the human leucocyte antigen D-related (HLA-DR) mean fluorescence intensity (MFI) of the DR^+ T_{reg} subset in healthy pregnancies and patients with gestational diabetes mellitus (GDM). (a–d) Quantitative changes of the DR^{low+} (a), DR^{high+} (b), DR^- (c) and $CD45RA^+$ (d) T_{reg} subsets in pregnancies affected by dietary-adjusted GDM (group 2, ◆) and insulin-dependent GDM (group 3, ◆) compared to healthy pregnancies (group 1, ♦). (e) The HLA-DR MFI of the DR^+ T_{reg} subset for healthy pregnancies (group 1, ♦), patients with dietary-adjusted GDM (group 2, ◆) and patients with insulin-dependent GDM (group 3, ◆).

position of the total $CD4^+CD127^{low+/-}CD25^+FoxP3^+$ T_{reg} pool with different T_{reg} subsets, we estimated its percentage of DR^{high+} (P4), DR^{low+} (P5), DR^- memory T_{regs} (P6) and naive $CD45RA^+$ T_{regs} (P7) (Fig. 1d) for healthy pregnancies (group 1), for patients with dietary-adjusted GDM (group 2) and patients with insulin-dependent GDM (groups 3). Additionally, we determined the HLA-DR mean fluorescence intensity (MFI) of the DR^+ memory T_{reg} subset (P8) for each participant. Table 2 and Fig. 4a–d depict the results for these measurements. In comparison to healthy pregnancies (group 1), we found a significantly decreased percentage of naive $CD45RA^+$ T_{regs} (Fig. 4d) for both patients with dietary-adjusted GDM (group 2) and patients with insulin-dependent GDM (group 3). Surprisingly, in patients with dietary-adjusted GDM (group 2), the percentage of DR^- memory T_{regs} (Fig. 4c) was increased significantly, while in patients with insulin-dependent GDM (group 3) the percentages of activated DR^{low+} (Fig. 4a) and DR^{high+} memory T_{regs} (Fig. 4b) were increased strongly compared to healthy pregnancies (group 1). As the HLA-DR MFI of total DR^+ memory T_{regs} was increased significantly in patients with insulin-dependent GDM (group 3), but not dietary-adjusted GDM patients, it seems that there is a stronger increase in the percentage of DR^{high+} memory T_{regs} compared to DR^{low+} memory T_{regs}. The direct statistical comparison between dietary- and insulin-treated pregnancies revealed a significantly decreased percentage of DR^- memory T_{regs} and a significantly increased percentage of DR^{high+} memory T_{regs} in insulin-dependent GDM patients. In summary, these results demonstrate clearly that both dietary-adjusted and

insulin-dependent GDM show a reduced percentage of naive CD45RA$^+$ T_{reg} cells and an increased percentage of CD45RA$^-$ memory T_{reg} cells. Obviously, there is an augmented conversion into DR$^-$ memory T_{regs} in patients with less pronounced disease, while patients with severe disease produce predominantly DR^{low+} and DR^{high+} memory T_{reg} cells. Similar results were obtained when dietary-treated and insulin-treated groups were pooled (groups 2 and 3, $n = 61$) and compared with the healthy control group (group 1, $n = 64$). The percentage of naive CD45RA$^+$ T_{regs} (median 35·8%, range 8·7–54·5%) was reduced significantly ($P < 0·001$), while the percentages of DR$^-$ memory T_{regs} (median 32·7%, range 21·8–44·9%, $P < 0·05$), DR^{low+} memory T_{regs} (median 24·0%, range 15·1–46·4%, $P < 0·001$) and DR^{high+} memory T_{regs} (median 3·3%, range 1·3–15·1%, $P < 0·001$) were increased significantly in pooled GDM patients compared to healthy pregnancies.

Discussion

In this exploratory study, we ascertained T_{reg} frequencies, their suppressive activity and their composition with distinct T_{reg} subsets (DR^{high+}, DR^{low+}, DR$^-$ memory T_{regs} and naive CD45RA$^+$ T_{regs}) in pregnancies affected by GDM compared to healthy pregnancies. Similar to other gestation-associated diseases (pre-eclampsia, preterm labour) [19,20], GDM was associated with decreased percentages of naive CD45RA$^+$ T_{regs}, but increased percentages of DR$^-$, DR^{low+} and DR^{high+} memory T_{regs}. Also notable is the fact that dietary-adjustable GDM was characterized by an increased percentage of DR$^-$ memory T_{regs}, while the more severe form of insulin-dependent GDM was associated with an increased percentage of the much more activated DR^{low+} and DR^{high+} memory T_{regs}. Such findings may suggest that according to the severity of the disease, an increased conversion of naive CD45RA$^+$ T_{regs} into less activated DR$^-$ memory T_{regs} occurs in the presence of dietary GDM, while an increased conversion into much more activated DR^{low+} and DR^{high+} memory T_{regs} occurs in the presence of insulin-dependent GDM.

Interestingly, similar findings are documented for non-regulatory peripheral T lymphocytes, such as CD4$^+$ T helper and CD8$^+$ cytotoxic T cell subsets. Compared to healthy pregnancies, a significantly reduced percentage of naive CD4$^+$CD45RA$^+$ T helper cells was found in patients with dietary-adjustable GDM and in patients with insulin-dependent GDM. In contrast, the percentages of CD4$^+$ CD45RO$^+$ memory T helper cells, the activated CD4$^+$CD25$^+$ and CD4$^+$HLA-DR$^+$ T helper cells were in fact increased significantly. In addition, significantly increased percentages of activated CD8$^+$CD25$^+$ and HLA-DR$^+$ cytotoxic T cells were also detected in these patient cohorts [22], indicating that both T_{resp} cells as well as T_{reg} cells are activated strongly in the presence of GDM.

Especially for T_{reg} cells, it was shown that the highly activated DR^{low+} and DR^{high+} memory T_{regs} have the highest suppressive activity within the total T_{reg} pool in normal healthy control subjects [23]. In particular, the DR^{high+} memory T_{regs} were found to be of potential importance for the suppressive activity of the total T_{reg} pool, as these cells were shown to be reduced significantly in kidney transplant patients with acute rejection, compared to non-rejecting patients [23,24]. Surprisingly, we found this relation to be reversed in pregnancy. In contrast to non-pregnant women, whose naive CD45RA$^+$ T_{regs} showed little suppressive activity, the CD45RA$^+$ T_{regs} showed the highest suppressive activity in pregnant women, while the DR$^+$ memory T_{regs} were less suppressive [25]. Therefore, changes in the composition of the total T_{reg} pool in the manner that the naive CD45RA$^+$ T_{regs} decrease, while the DR^{low+} DR^{high+} memory T_{regs} increase, may cause a significant loss of the suppressive activity of the total T_{reg} pool in pregnant women. Meanwhile, such changes are demonstrated for many gestation-associated diseases, such as pre-eclampsia and preterm labour, and may explain the loss of the suppressive activity of the T_{reg} pool in these patients. At present, it is not known which immunological mechanisms cause such differences in the suppressive activity of the individual T_{reg} subsets between pregnant and non-pregnant women.

Our findings may suggest that the normal maternal immune suppression is less effective in GDM patients, due presumably to the fact that the existing T_{reg} pool in GDM patients is strongly activated, but not capable of fulfilling its suppressive activity. However, the exact mechanisms through which such immune activation and inflammation lead to glucose intolerance in pregnancy are still unclear. There are some data which demonstrate that inflammasome activation in adipose tissue interferes with the insulin signalling pathway, leading to insulin resistance [14]. Meanwhile, it is also known that there is a strong relationship between inflammatory signals and rising serum pentraxin 3 (PTX3) levels [26]. PTX 3 is known as a marker of vascular inflammation and atherosclerotic plaque formation, and therefore it is considered as a strong prognostic factor of mortality after myocardial infarction [27]. A significantly increased rise of PTX3 was also found in GDM patients during oral glucose tolerance test (OGTT). In addition, there was a negative correlation of PTX3 and insulin sensitivity in these patients [28]. These data suggest a link between this inflammatory cardiovascular risk marker and markers for glucose intolerance and insulin resistance in pregnant women. Also, recent data showed that PTX3 is elevated significantly in patients affected by pre-eclampsia [29,30]. Such findings propose that inflammatory events, due presumably to aberrant immune responses, may be involved in the pathogenesis of both GDM and pre-eclampsia [31].

However, our current data do not yet allow the conclusion that the observed changes in the composition of the total T_{reg} pool and the resulting decrease of its suppressive activity is responsible for the amplification of inflammatory

processes observed in GDM patients. Such mechanisms may not be the cause of the disease, but may simply reflect the consequences of the disease rather than a pathway leading to GDM. In addition, it should be noted that our study is based on exploratory analyses and thus the results have to be interpreted with care. As a validation cohort is missing, because it is ethically not acceptable to treat healthy pregnant women with insulin, the data are somewhat suggestive and require further validation. Notably, in a murine obesity model for GDM, it has been demonstrated that GDM leads to chronic hypoxia stress and an excessive inflammatory response in the murine placenta, thus suggesting a severe dysregulation of the immune tolerance network under these conditions [32]. Hence, additional investigations concerning the role of immune activation and inflammation in the pathogenesis of GDM are necessary to ultimately clarify whether an aberrant T_{reg} cell compartment is involved in the pathogenesis of GDM, and also presumably in the development of related complications, such as type 2 diabetes, metabolic syndrome and cardiovascular diseases.

Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft grant STE 885/3-2 (to A. S.). We would like to thank M. Hoerner, C. Schneider and the nursing staff of the delivery room and the ultrasound section of the Department of Obstetrics and Gynecology, University of Heidelberg, for arranging the collection of blood samples.

Disclosure

None of the authors have any conflicts of interest related to this manuscript.

Author contributions

A. S., L. S., D. R. and E. S. designed the study, D. R., A. K. and L. S. performed the study, K. M., L. U., H. F. and C. S. contributed important methods and patients, L. S., D. R., J. S. and A. S. collected and analysed the data, L. S. and A. S. wrote the paper. All authors contributed to the final version of the paper and approved it.

References

1 Crowther CA, Hiller JE, Moss JR, McPhee AJ, Jeffreis WS, Robinson JS for the Australian Carbohydrate Intolerance Study in Pregnant Women (ACHOIS) Trial Group. Effect of treatment of gestational diabetes mellitus on pregnancy outcomes. N Engl J Med 2005; **352**:2477–86.

2 The HAPO Study Cooperative Research Group. Hyperglycemia and adverse pregnancy outcomes. N Engl J Med 2008; **358**:1991–2002.

3 Kim C, Newton KM, Knopp RH. Gestational diabetes and the incidence of type 2 diabetes: a systemic review. Diabetes Care 2002; **25**:1862–8.

4 Tam WH, Ma RC, Yang X *et al*. Glucose intolerance and cardiometabolic risk in adolescents exposed to maternal gestational diabetes: a 15 year follow-up study. Diabetes Care 2010; **33**:1382–4.

5 Van Leeuwen M, Louwerse MD, Opmeer BC *et al*. Glucose challenge test for detecting gestational diabetes mellitus: a systematic review. Br J Obstet Gynaecol 2012; **119**:393–401.

6 Harlev A, Wiznitzer A. New insights on glucose pathophysiology in gestational diabetes and insulin resistance. Curr Diab Rep 2010; **10**:242–47.

7 Kautzky-Willer A, Prager R, Waldhausl W *et al*. Pronounced insulin resistance and inadequate beta-cell insulin secretion characterize lean gestational diabetes during and after pregnancy. Diabetes Care 1997; **20**:1717–23.

8 Sladek R, Rocheleau G, Rung J *et al*. A genome-wide association study identifies novel risk loci for type two diabetes. Nature 2007; **445**:881–5.

9 Mao H, Li Q, Gao S. Meta-analysis of the relationship between common type 2 diabetes risk gene variants with gestational diabetes mellitus. PLOS ONE 2012; **7**:e45882.

10 Enquobahrie DA, Williams MA, Qiu C, Meller M, Sorensen TK. Global placental gene expression in gestational diabetes mellitus. Am J Obstet Gynecol 2009; **200**:206.e1–206.e13.

11 Atègbo JM, Grissa O, Yessoufou A *et al*. Modulation of adipokines and cytokines in gestational diabetes and macrosomia. J Clin Endocrinol Metab 2006; **91**:4137–43.

12 Richardson AC, Carpenter MW. Inflammatory mediators in gestational diabetes mellitus. Obstet Gynecol Clin North Am 2007; **34**:213–24.

13 Steinborn A, Saran G, Schneider A, Fersis N, Sohn C, Schmitt E. The presence of gestational diabetes is associated with increased detection of anti-HLA-class II antibodies in the maternal circulation. Am J Reprod Immunol 2006; **56**:124–34.

14 Lappas M. Activation of inflammasomes in adipose tissue of women with gestational diabetes. Mol Cell Endocrinol 2014; **382**:74–83.

15 Sakaguchi S, Miyara M, Costantino C, Hafler DA. $FOXP3^+$ regulatory T cells in the human immune system. Nat Rev Immunol 2010; **10**:490–500.

16 Aluvihare VR, Kallikourdis M Betz AG. Regulatory T cells mediate maternal tolerance to the fetus. Nat Immunol 2004; **5**:266–71.

17 Booth NJ, McQuaid AJ, Sobande T *et al*. Different proliferative potential and migratory characteristics of human $CD4^+$ regulatory T cells that express either CD45RA or CD45RO. J Immunol 2010; **184**:4317–26.

18 Baecher-Allan C, Wolf E, Hafler D. MHC class II expression identifies functionally distinct human regulatory T cells. J Immunol 2006; **176**:4622–31.

19 Schober L, Radnai D, Schmitt E, Mahnke K, Sohn C, Steinborn A. Term and preterm labor: decreased suppressive activity and changes in composition of the regulatory T-cell pool. Immunol Cell Biol 2012; **90**:935–44.

20 Steinborn A, Schmitt E, Kisielewicz A *et al*. Pregnancy-associated diseases are characterized by the composition of the systemic regulatory T cell (T_{reg}) pool with distinct subsets of T_{regs}. Clin Exp Immunol 2012; **167**:84–98.

21 Wendland EM, Torloni MR, Falavigna M *et al*. Gestational diabetes and pregnancy outcomes – a systematic review of the World Health Organization (WHO) and the International Association of Diabetes in Pregnancy Study Groups (IADPSG) diagnostic criteria. BMC Pregnancy Childbirth 2012; **12**:23.
22 Mahmoud F, Abul H, Omu A, Haines D. Lymphocyte subpopulations in gestational diabetes. Am J Reprod Immunol 2005; **53**:21–9.
23 Schaier M, Seissler N, Schmitt E *et al*. DR(high+)CD45RA(–)-Tregs potentially affect the suppressive activity of the total Treg pool in renal transplant patients. PLOS ONE 2012; **7**:e34208.
24 Schaier M, Seissler N, Becker LE *et al*. The extent of HLA-DR expression on HLA-DR(+) Tregs allows the identification of patients with clinically relevant borderline rejection. Transpl Int 2013; **26**:290–9.
25 Schlossberger V, Schober L, Rehnitz J *et al*. The success of assisted reproduction technologies in relation to composition of the total regulatory T cell (Treg) pool and different Treg subsets. Hum Reprod 2013; **28**:3062–73.
26 Garlanda C, Bottazzi B, Bastone A, Mantovani A. Pentraxins at the crossroads between innate immunity, inflammation, matrix deposition and female fertility. Ann Rev Immunol 2005; **23**:337–66.
27 Latini R, Maggioni AP, Peri G *et al*. Prognostic significance of the long pentraxin PTX3 in acute myocardial infarction. Circulation 2004; **110**:2349–54.
28 Todoric J, Handisurya A, Knapp B, Tura A, Pacini G, Kautzky-Willer A. Relationship of pentraxin 3 with insulin sensitivity in gestational diabetes. Eur J Clin Invest 2013; **43**: 341–9.
29 Castiglioni MT, Scavini M, Cavallin R *et al*. Elevation of plasma levels of the long pentraxin 3 proceeds preeclampsia in pregnant patients with type 1 diabetes. Autoimmunity 2009; **42**:296–8.
30 Zhou P, Luo X, Qi HB *et al*. The expression of pentraxin 3 and tumor necrosis factor-alpha is increased in preeclamptic placental tissue and maternal serum. Inflamm Res 2012; **61**:1005–12.
31 Laresgoiti-Servitje E, Gómez-Lopéz N, Olson DM. An immunological insight into the origins of pre-eclampsia. Hum Reprod Update 2010; **16**:510–24.
32 Li HP, Chen X, Li MQ. Gestational diabetes induces chronic hypoxia stress and excessive inflammatory response in murine placenta. Int J Clin Exp Pathol 2013; **6**:650–9.